Exhibit A

ZAP Express cDNA Synthesis Kit and ZAP Express cDNA Gigapack III Gold Cloning Kit

INSTRUCTION MANUAL

Catalog #200403 (ZAP Express cDNA Synthesis Kit) and
#200451 (ZAP Express cDNA Gigapack III Gold Cloning Kit)

Caution ***DO NOT*** *substitute the components in this kit with components from another kit. Component substitution may result in lower efficiency library construction.*

Revision B.01

For In Vitro Use Only
200403-12

LIMITED PRODUCT WARRANTY

This warranty limits our liability to replacement of this product. No other warranties of any kind, express or implied, including without limitation, implied warranties of merchantability or fitness for a particular purpose, are provided by Agilent. Agilent shall have no liability for any direct, indirect, consequential, or incidental damages arising out of the use, the results of use, or the inability to use this product.

ORDERING INFORMATION AND TECHNICAL SERVICES

United States and Canada

Agilent Technologies
Stratagene Products Division
11011 North Torrey Pines Road
La Jolla, CA 92037
Telephone (858) 373-6300
Order Toll Free (800) 424-5444
Technical Services (800) 894-1304
Internet techservices@agilent.com
World Wide Web www.stratagene.com

Europe

Location	Telephone	Fax	Technical Services
Austria	0800 292 499	0800 292 496	0800 292 498
Belgium	00800 7000 7000	00800 7001 7001	00800 7400 7400
	0800 15775	0800 15740	0800 15720
France	00800 7000 7000	00800 7001 7001	00800 7400 7400
	0800 919 288	0800 919 287	0800 919 289
Germany	00800 7000 7000	00800 7001 7001	00800 7400 7400
	0800 182 8232	0800 182 8231	0800 182 8234
Netherlands	00800 7000 7000	00800 7001 7001	00800 7400 7400
	0800 023 0446	+31 (0)20 312 5700	0800 023 0448
Switzerland	00800 7000 7000	00800 7001 7001	00800 7400 7400
	0800 563 080	0800 563 082	0800 563 081
United Kingdom	00800 7000 7000	00800 7001 7001	00800 7400 7400
	0800 917 3282	0800 917 3283	0800 917 3281

All Other Countries

Please contact your local distributor. A complete list of distributors is available at www.stratagene.com.

ZAP Express cDNA Synthesis Kit and ZAP Express cDNA Gigapack III Gold Cloning Kit

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ZAP Express cDNA Synthesis Kit and ZAP Express cDNA Gigapack III Gold Cloning Kit

MATERIALS PROVIDED

Materials provided[a]	Quantity	
	Catalog #200403	**Catalog #200451**
cDNA Synthesis Kit (Catalog #200401)	All reagents and labware	All reagents and labware
ZAP Express vector digested with *Eco*R I and *Xho* I, CIAP treated*[,b]	12 µg	12 µg
pBR322 test insert digested with *Sal* I (compatible with *Xho* I) and *Eco*R I	2.5 µg	2.5 µg
XL1-Blue MRF′ strain[c]	0.5-ml bacterial glycerol stock	0.5-ml bacterial glycerol stock
XLOLR strain[c]	0.5-ml bacterial glycerol stock	0.5-ml bacterial glycerol stock
ExAssist interference-resistant helper phage[d,e]	1 ml	1 ml
R408 Interference-Resistant Helper Phage[e,f]	1 ml	1 ml
Gigapack III Gold packaging extract[g]	—	11 × 25 µl
λcI857 *Sam*7 wild-type lambda control DNA[h]	—	1.05 µg
VCS257 host strain[i]	—	1-ml bacterial glycerol stock

[a] Enough reagents are included to generate five vector-ligated constructs. Depending on the cloning efficiencies achieved, purchase of additional Gigapack III Gold packaging extract may be necessary.

[b] On arrival, store the ZAP Express vector at –20°C. After thawing, aliquot and store at –20°C. Do not pass through more than two freeze–thaw cycles. For short-term storage, store at 4°C for 1 month.

[c] Use the XLOLR strain for plating excised phagemids and the XL1-Blue MRF′ strain for all other manipulations. For host strain shipping and storage conditions, see *Bacterial Host Strains*.

[d] The titer of the ExAssist interference-resistant helper phage is ~1.0 × 10^{10} pfu/ml. This supercoiled single-stranded DNA migrates at ~5 kb on an agarose gel. The ExAssist helper phage is recommended for excision of the pBK-CMV phagemid vector from the ZAP Express vector. It should not be used for single-stranded rescue, because this f1 helper phage possesses α-complementing β-galactosidase sequences which may interfere with sequencing or site-directed mutagenesis where oligonucleotide primers hybridize to β-galactosidase sequences (e.g., M13–20 primer).

[e] Retiter after 1 month. (Take care not to contaminate the Uni-ZAP XR vector with this high-titer filamentous helper phage.) Store at –80°C.

[f] The titer of the R408 Interference-Resistant Helper Phage is ~7.5 × 10^{10} pfu/ml. This supercoiled single-stranded DNA migrates at ~4 kb on an agarose gel. The R408 Interference-Resistant Helper Phage is recommended for single-stranded rescue (see *Appendix V: Recovery of Single-Stranded DNA from Cells Containing the pBK-CMV Phagemid Vector*).

[g] Gigapack III packaging extract is very sensitive to slight variations in temperature. Storing the packaging extracts at the bottom of a –80°C freezer directly from the dry ice shipping container is required in order to prevent a loss of packaging efficiency. Transferring tubes from one freezer to another may also result in a loss of efficiency. **Do not allow the packaging extracts to thaw!** Do not store the packaging extracts in liquid nitrogen as the tubes may explode.

[h] The λcI857 *Sam*7 wild-type lambda control DNA is shipped frozen and should be stored at –80°C immediately on receipt.

[i] The VCS257 host strain, included for plating the λcI857 *Sam*7 wild-type lambda control DNA, is shipped as a frozen bacterial glycerol stock (see *Bacterial Host Strains* for additional storage instructions) and should also be stored at –80°C immediately on receipt. This control host strain is a derivative of DP50 *supF* and should be used only when plating the packaged lambda control DNA. The lambda control DNA used with Gigapack III Gold packaging extract requires a *supF* mutation in the bacterial host to plate efficiently.

* U.S. Patent Nos. 5,128,256 and 5,286,636.

Revision B.01

Caution ***DO NOT*** *substitute the reagents listed below with reagents from another kit. Component substitution may result in lower efficiency library construction.*

Reagents and Labware Provided with the cDNA Synthesis Kit

Reagents and labware provided[a]	Quantity	Storage temperature
First-strand reagents		
AccuScript reverse transcriptase (AccuScript RT)	15 µl	–20°C
RNase Block Ribonuclease Inhibitor (40 U/µl)	200 U	–20°C
First-strand methyl nucleotide mixture (10 mM dATP, dGTP, and dTTP plus 5 mM 5-methyl dCTP)	15 µl	–20°C
First-strand buffer (10×)	75 µl	–20°C
Linker–primer (1.4 µg/µl)	10 µl	–20°C
Test poly(A)$^+$ RNA (0.2 µg/µl)	5 µg	–20°C
Diethylpyrocarbonate (DEPC)-treated water	500 µl	–20°C
Second-strand reagents		
Second-strand buffer (10×)	150 µl	–20°C
Second-strand dNTP mixture (10 mM dATP, dGTP, and dTTP plus 26 mM dCTP)	30 µl	–20°C
Escherichia coli RNase H (1.5 U/µl)	15 U	–20°C
Escherichia coli DNA polymerase I (9.0 U/µl)	500 U	–20°C
Sodium acetate (3 M)	250 µl	–20°C
Blunting reagents		
Blunting dNTP mixture (2.5 mM dATP, dGTP, dTTP, and dCTP)	115 µl	–20°C
Cloned *Pfu* DNA polymerase (2.5 U/µl)	25 U	–20°C
Ligation reagents		
*Eco*R I adapters (0.4 µg/µl)	18 µg	–20°C
Ligase buffer[b,c] (10×)	250 µl	–20°C
rATP[b] (10 mM)	100 µl	–20°C
T4 DNA ligase[b] (4 U/µl)	140 U	–20°C
Phosphorylation reagents		
T4 polynucleotide kinase (5 U/µl)	50 U	–20°C
Ligase buffer[b,c] (10×)	250 µl	–20°C
rATP[b] (10 mM)	100 µl	–20°C
Xho I digestion reagents		
Xho I (40 U/µl)	600 U	–20°C
Xho I buffer supplement	250 µl	–20°C
Column reagents and labware		
Connecting tubing[d] (1/8-inch i.d., 3/16-inch o.d., and 1/32-inch wall)	1 × 4 cm	Room temperature or 4°C
Sepharose® CL-2B gel filtration medium[d]	10 ml	4°C
Column-loading dye[c,d]	17.5 µl	4°C
STE buffer[c] (10×)	10 ml	4°C

[a] Enough reagents are included to generate five vector-ligated constructs.

[b] These reagents are used more than once in the reaction.

[c] See *Preparation of Media and Reagents*.

[d] The column reagents and labware are shipped separately at 4°C.

STORAGE CONDITIONS

Sepharose® CL-2B Gel Filtration Medium: 4°C

Column-Loading Dye: 4°C

ZAP Express Vector: –20°C

Test Insert: –20°C

Helper Phage: –80°C

Bacterial Glycerol Stocks: –80°C

Packaging Extracts: –80°C

Other Reagents: –20°C

ADDITIONAL MATERIALS REQUIRED

Certain reagents recommended in this instruction manual are potentially dangerous and present the following hazards: chemical (DEPC, phenol, chloroform, and sodium hydroxide), radioactive (^{32}P-labeled radioisotope), or physical (high-voltage electrophoresis systems). The researcher is advised to take proper precautions and care with these hazards and to follow the safety recommendations from each respective manufacturer.

Reagents and Solutions

Phenol–chloroform [1:1 (v/v)] and chloroform

Note *Do not use the low-pH phenol from the Stratagene RNA Isolation Kit because this phenol is acidic and may denature the DNA.*

Ethanol [70%, 80%, and 100% (v/v)]
Gigapack III Gold packaging extract (for Catalog #200403 only) [Catalog #200201 (Gigapack III Gold-4), #200202 (Gigapack III Gold-7), and #200203 (Gigapack III Gold-11)]
Sterile distilled water (dH_2O)
α-^{32}P-labeled deoxynucleotide (800 Ci/mmol) ([^{32}P]dATP, [^{32}P]dGTP, or [^{32}P]dTTP may be used; do not use [^{32}P]dCTP)

Equipment

Ribonuclease (RNase)-free microcentrifuge tubes and pipet tips
Disposable plastic 10-ml syringes, sterile (e.g., B-D® 10-cc syringe with a Luer Lok® tip or equivalent)
Disposable 18-guage, 1½-inch needles, sterile (e.g., B-D® *PrecisionGlide*® needle or equivalent)
Disposable plastic 1-ml pipets, negatively graduated and sterile [e.g., Falcon® 7520 1-ml serological pipet (1 ml) or equivalent]
Pasteur pipet
Portable radiation monitor (Geiger counter)
Water baths (4°, 8°, 12°, 16°, 42°, and 70°C)
Microcentrifuge
Micropipet and micropipet tips
Vacuum evaporator
Incubator (37°C)
14-ml BD Falcon polypropylene round-bottom tubes

NOTICES TO PURCHASER

CMV Promoter

The use of the CMV Promoter is covered under U.S. Patent Nos. 5,168,062 and 5,385,839 owned by the University of Iowa Research Foundation and licensed FOR RESEARCH USE ONLY. For further information, please contact UIRF at 319-335-4546.

ZAP Express Vector

The ZAP Express vector is covered by United States Patent Nos. 5,128,256 and 5,286,636. The purchase of this vector includes a limited, nonexclusive license under such patent rights to use the vector for the cloning, expression, and characterization of genes. This license does not grant rights to (1) use the ZAP Express vector for the reproduction, amplification, or modification of the vector; (2) offer the ZAP Express vector or any derivative thereof for resale; (3) distribute or transfer the ZAP Express vector or any derivative thereof to any third party; or (4) incorporate the ZAP Express vector or any derivative thereof in any genomic or cDNA library for resale, distribution, or transfer to any third party. No other license, express, implied, or by estoppel, is granted. For information concerning the availability of licenses to reproduce and/or modify the ZAP Express vector, please contact the Technical Services Department at 1-800-424-5444.

This product is for research purposes only and must be used in accordance with NIH guidelines for recombinant DNA.

BACKGROUND

Complementary DNA (cDNA) libraries represent the information encoded in the messenger RNA (mRNA) of a particular tissue or organism. RNA molecules are exceptionally labile and difficult to amplify in their natural form. For this reason, the information encoded by the RNA is converted into a stable DNA duplex (cDNA) and then is inserted into a self-replicating lambda vector. Once the information is available in the form of a cDNA library, individual processed segments of the original genetic information can be isolated and examined with relative ease.

INTRODUCTION

The ZAP Express synthesis kit uses a hybrid oligo(dT) linker–primer that contains an *Xho* I restriction site. Messenger RNA is primed in the first-strand synthesis with the linker–primer and is reverse-transcribed using AccuScript reverse transcriptase and 5-methyl dCTP.

AccuScript reverse transcriptase (AccuScript RT) is a novel Moloney murine leukemia virus reverse transcriptase (MMLV-RT) derivative combined with a proofreading 3'-5' exonuclease. AccuScript reverse transcriptase delivers the highest reverse-transcription accuracy while promoting full length cDNA synthesis. AccuScript reverse transcriptase delivers greater than three-fold higher accuracy compared to leading reverse transcriptases, representing a significant advancement in cDNA synthesis accuracy. These advantages make AccuScript RT the enzyme of choice for applications involving the preparation of accurate, full-length, cDNA transcripts, including first-strand cDNA synthesis and library construction.

The use of 5-methyl dCTP during first-strand synthesis hemimethylates the cDNA, which protects the cDNA from digestion with certain restriction endonucleases such as *Xho* I. Therefore, on *Xho* I digestion of the cDNA, only the unmethylated site within the linker–primer is cleaved.

Hemimethylated DNA introduced into an McrA$^+$ McrB$^+$ strain would be subject to digestion by the *mcrA* and *mcrB* restriction systems. Therefore, it is necessary to initially infect an McrA$^-$ McrB$^-$ strain (e.g., XL1-Blue MRF′ strain supplied with the ZAP Express vector) when using the ZAP Express cDNA synthesis kit. After passing the library through XL1-Blue MRF′ cells, the DNA is no longer hemimethylated and can be grown on McrA$^+$ McrB$^+$ strains (e.g., XL1-Blue strain).

Note *Use high-efficiency Gigapack III Gold packaging extract, since this packaging extract is McrA$^-$, McrB$^-$, and Mrr$^-$. Other commercially available packaging extracts can destroy hemimethylated DNA, therefore producing low-titer libraries.*

The yield, length, and accuracy of cDNA transcripts is enhanced with the use of AccuScript RT, an engineered version of the Moloney murine leukemia virus reverse transcriptase combined with a proofreading 3'-5' exonuclease. First-strand cDNA synthesis begins when AccuScript RT, in the presence of nucleotides and buffer, finds a template and a primer. The template is mRNA and the primer is a 50-base oligonucleotide with the following sequence:

5´-GAGAGAGAGAGAGAGAGAGAACTAGT**CTCGAG**TTTTTTTTTTTTTTTTTT-3´
"GAGA" Sequence *Xho* I Poly(dT)

This oligonucleotide was designed with a "GAGA" sequence to protect the *Xho* I restriction enzyme recognition site and an 18-base poly(dT) sequence. The restriction site allows the finished cDNA to be inserted into the ZAP Express vector[1] in a sense orientation (*Eco*R I–*Xho* I) with respect to the *lacZ* promoter. The poly(dT) region binds to the 3´ poly(A) region of the mRNA template, and AccuScript RT begins to synthesize the first-strand cDNA.

The nucleotide mixture for the first strand contains normal dATP, dGTP, and dTTP plus the analog 5-methyl dCTP. The complete first strand will have a methyl group on each cytosine base, which will protect the cDNA from restriction enzymes used in subsequent cloning steps.

During second-strand synthesis, RNase H nicks the RNA bound to the first-strand cDNA to produce a multitude of fragments, which serve as primers for DNA polymerase I. DNA polymerase I "nick-translates" these RNA fragments into second-strand cDNA. The second-strand nucleotide mixture has been supplemented with dCTP to reduce the probability of 5-methyl dCTP becoming incorporated in the second strand. This ensures that the restriction sites in the linker–primer will be susceptible to restriction enzyme digestion. The uneven termini of the double-stranded cDNA are nibbled back or filled in with cloned *Pfu* DNA polymerase, and *Eco*R I adapters are ligated to the blunt ends. The adapters have the sequence shown below.

5´-OH-AATTCGGCACGAGG-3´
3´-GCCGTGCTCCp-5´

These adapters are composed of 10- and 14-mer oligonucleotides, which are complementary to each other with an *Eco*R I cohesive end. The 10-mer oligonucleotide is phosphorylated, which allows it to ligate to other blunt termini available in the form of cDNA and other adapters. The 14-mer oligonucleotide is kept dephosphorylated to prevent it from ligating to other cohesive ends. After adapter ligation is complete and the ligase has been heat inactivated, the 14-mer oligonucleotide is phosphorylated to enable its ligation to the dephosphorylated vector arms.

The *Xho* I digestion releases the *Eco*R I adapter and residual linker–primer from the 3′ end of the cDNA. These two fragments are separated on a drip column containing Sepharose® CL-2B gel filtration medium. The size-fractionated cDNA is then precipitated and ligated to the ZAP Express vector.

The lambda library is packaged in a high-efficiency system such as Gigapack III Gold packaging extract[2] and is plated on the *E. coli* cell line XL1-Blue MRF′.[3] **Since most *E. coli* strains digest DNA containing 5′-methyl dCTP, it is important to plate on this McrA⁻ McrB⁻ strain.**

Note *An outline of the ZAP Express cDNA synthesis kit protocol is provided (see Figure 1). If you plan to be away from the project for 1 or 2 days, try to schedule the synthesis such that the cDNA remains in the ligation reaction. Even though the majority of ligation is complete in the time recommended by the procedure, the provided ligase is extremely active and will continue to find and ligate available ends. Although most investigators wish to produce their cDNA libraries as rapidly as possible, it is important to remember that extended ligations and overnight precipitations can increase the yield.*

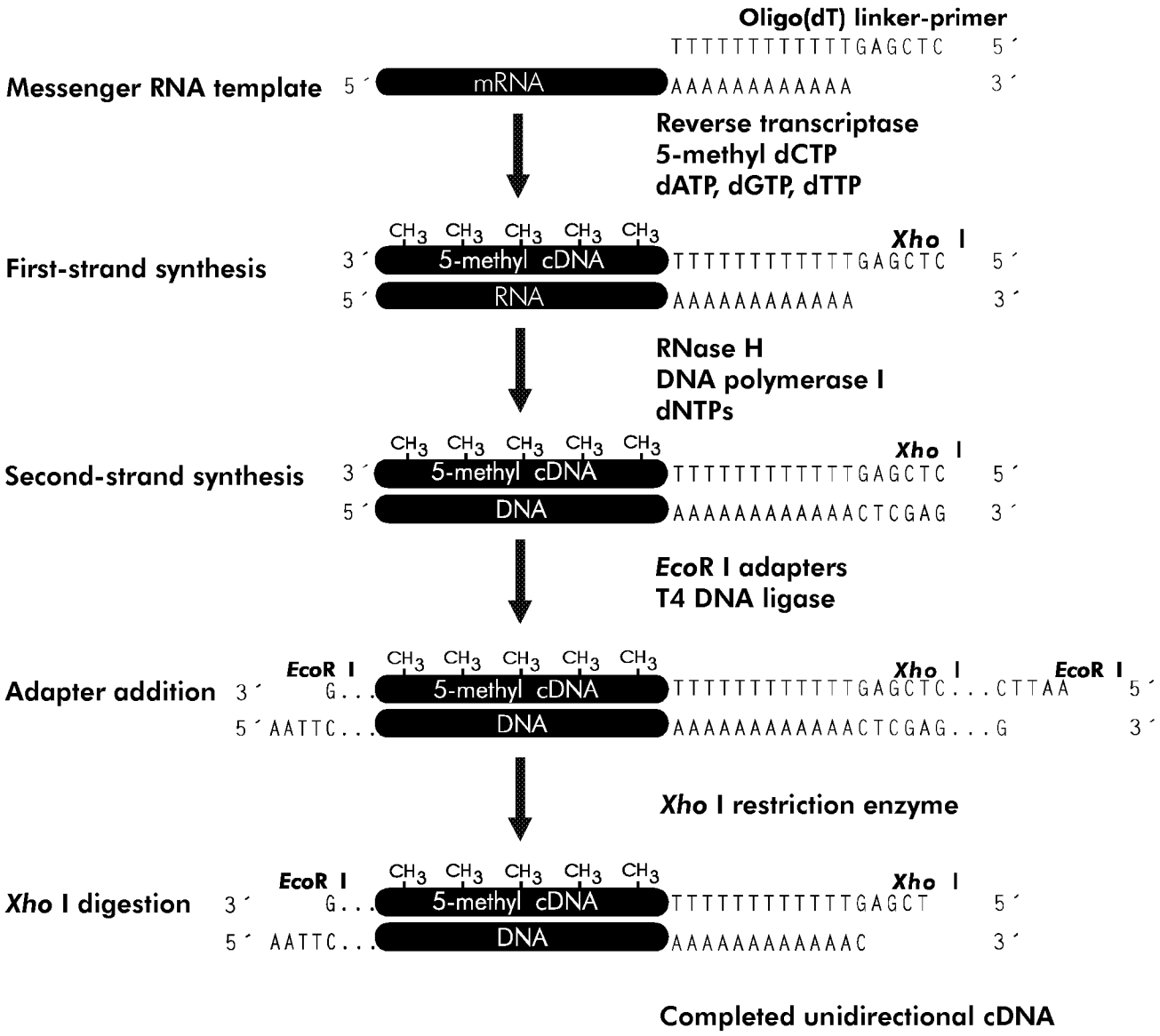


FIGURE 1 cDNA synthesis flow chart.

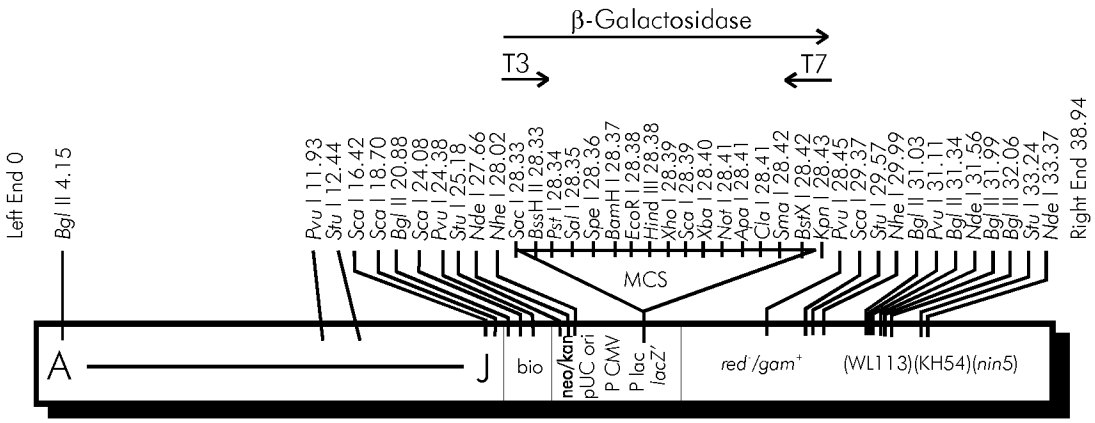


FIGURE 2 Map of the ZAP Express vector.

GENERAL VECTOR DESCRIPTION

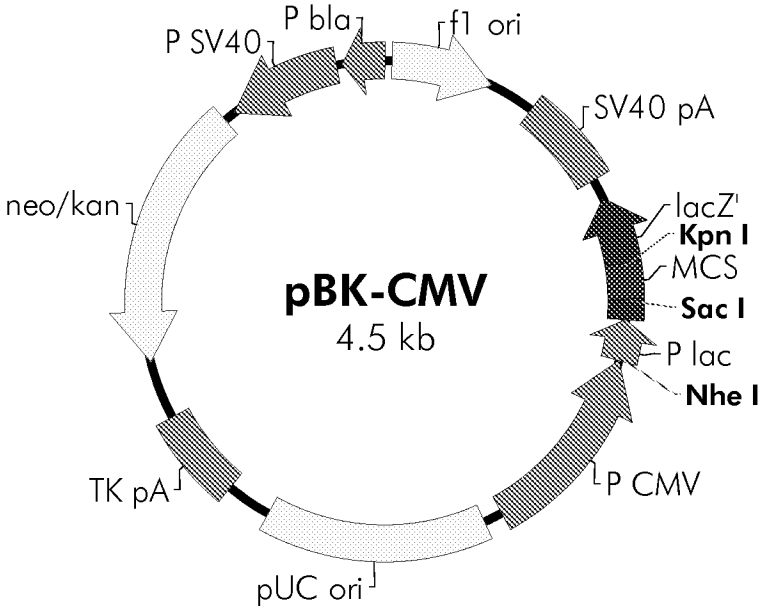
The ZAP Express vector (Figure 2) allows both eukaryotic and prokaryotic expression, while also increasing both cloning capacity and the number of unique lambda cloning sites.[4] The ZAP Express vector has 12 unique cloning sites which will accommodate DNA inserts from 0 to 12 kb in length. The 12 unique cloning sites are *Apa* I, *Bam*H I, *Eco*R I, *Hin*d III, *Kpn* I, *Not* I, *Sac* I, *Sal* I, *Sma* I, *Spe* I, *Xba* I, and *Xho* I. Inserts cloned into the ZAP Express vector can be excised out of the phage in the form of the kanamycin-resistant pBK-CMV phagemid vector (Figure 3) by the same excision mechanism found in the Lambda ZAP vectors.[1, 4, 5]

Clones in the ZAP Express vector can be screened with either DNA probes or antibody probes, and in vivo rapid excision of the pBK-CMV phagemid vector allows insert characterization in a plasmid system. The polylinker of pBK-CMV phagemid vector has 17 unique cloning sites flanked by T3 and T7 promoters and has 3 standardized primer sites for DNA sequencing. The plasmid has the bacteriophage f1 origin of replication allowing rescue of ssDNA, which can be used for DNA sequencing or site-directed mutagenesis. Unidirectional deletions can be made using exonuclease III and mung bean nuclease by taking advantage of the unique positioning of 5′ and 3′ restriction sites. Transcripts made from the T3 and T7 promoters generate riboprobes useful in Southern and Northern blotting, and the *lacZ* promoter may be used to drive expression of fusion proteins suitable for Western blot analysis or protein purification.

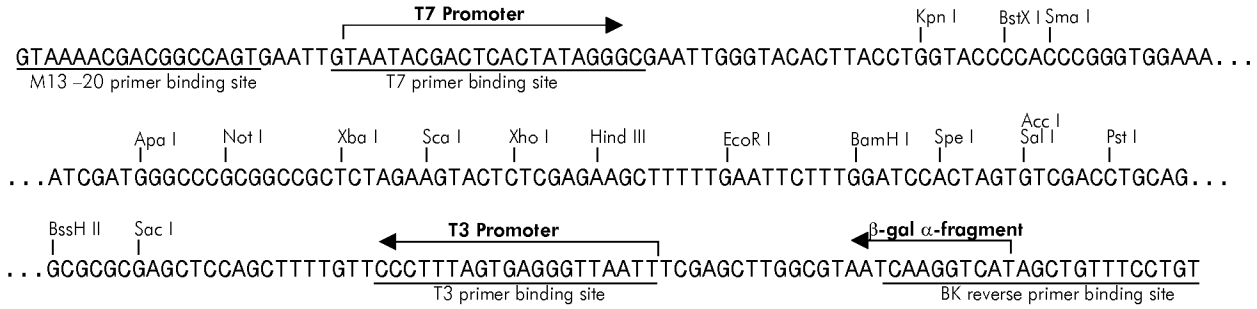
Eukaryotic expression of inserts is driven by the cytomegalovirus (CMV) immediate early (IE) promoter with the SV40 transcription terminator and polyadenylation signal.

Stable selection of clones in eukaryotic cells is made possible by the presence of the neomycin- and kanamycin-resistance gene, which is driven by the SV40 early promoter with TK transcription polyadenylation signals to render transfectants resistant to G418 (geneticin).

pBK-CMV Vector Map



pBK-CMV Multiple Cloning Site Region (sequence shown 952–1196)



Feature	Nucleotide Position
f1 origin of ss-DNA replication	24–330
SV40 polyA signal	469–750
β-galactosidase α-fragment coding sequence (lacZ′)	812–1183
multiple cloning site	1015–1122
lac promoter	1184–1305
CMV promoter	1306–1895
pUC origin of replication	1954–2621
HSV-thymidine kinase (TK) polyA signal	2760–3031
neomycin/kanamycin resistance ORF	3209–4000
SV40 promoter	4035–4373
bla promoter	4392–4518

FIGURE 3 The pBK-CMV phagemid vector. The complete sequence and list of restriction sites are available at *www.stratagene.com*.

BACTERIAL HOST STRAINS

Host Strain Genotypes

Host strain	Genotype
XL1-Blue MRF′ strain	Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F′ *proAB lacI*^{q}*Z*Δ*M15* Tn*10* (Tet^{r})]
XLOLR strain[a]	Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 thi-1 recA1 gyrA96 relA1 lac* [F′ *proAB lacI*^{q}*Z*Δ*M15* Tn*10* (Tet^{r})] Su^{-} (nonsuppressing) λ^{r} (lambda resistant)

[a] Use the XLOLR strain for excision only.

XL1-Blue MRF′ Bacterial Strain Description

The RecA^{-} *E. coli* host strain XL1-Blue MRF′ is supplied with the ZAP Express cDNA synthesis kit. Because the pBK-CMV phagemid vector does not require a *supF* genotype, the amplified library grows very efficiently on the XL1-Blue MRF′ strain. In addition, use of the correct host strain is important when working with the pBK-CMV phagemid vector as the F′ episome present in the XL1-Blue MRF′ strain serves three purposes.

First, the Δ*M15 lacZ* gene present on the F′ episome is required for the β-galactosidase-based nonrecombinant selection strategy. When cDNA is present in the polylinker, expression from the *lacZ* gene is disrupted and white plaques are produced. In contrast, without insert in the polylinker, the amino terminus of β-galactosidase is expressed and nonrecombinants can be scored visually by the presence of blue plaques. To produce an enzymatically active β-galactosidase protein, two domains are required: the α-region expressed by the vector and the Δ*M15 lacZ* domain expressed by the F′ episome. These two domains fold to form a functional protein, the α-region complementing the missing amino acids resulting from the Δ*M15* mutation. Therefore, in order to utilize the nonrecombinant selection strategy, the correct host strain must be used to produce a functional β-galactosidase protein.

Second, the F′ episome expresses the genes forming the F′ pili found on the surface of the bacteria. Without pili formation, filamentous phage (i.e., M13 or f1) infection could not occur. Because the conversion of a recombinant ZAP Express clone to a pBK-CMV phagemid vector requires superinfection with a filamentous helper phage, the F′ episome is required for in vivo excision (see *In Vivo Excision of the pBK-CMV Phagemid Vector from the ZAP Express Vector*).

Third, the F′ episome contains the *lac* repressor (*lacI*^{q} gene), which blocks transcription from the *lacZ* promoter in the absence of the inducer isopropyl-1-thio-β-D-galactopyranoside (IPTG). This repressor is important for controlling expression of fusion proteins that may be toxic to *E. coli*. Because the presence of the *lacI*^{q} repressor in the *E. coli* host strain can potentially increase the representation or completeness of the library, XL1-Blue MRF′ is useful for screening the amplified library.

Note *The strains used for the Lambda gt11 vector (i.e., Y1088, Y1089, and Y1090) are not suitable for use with the ZAP Express vector. These strains contain the plasmid pMC9, a pBR322 derivative, which contains many of the same sequences as those found in the phagemid portion of the ZAP Express vector. The SURE strain and the SOLR strain are not compatible with the ZAP Express system, since these strains contain the kanamycin-resistance gene found in the pBK-CMV phagemid vector. Using these strains with the ZAP Express vector could result in recombination between the homologous sequences.*

Recommended Media

Host strain	Agar plates and liquid medium for bacterial streak and glycerol stock	Liquid medium for bacterial cultures prior to phage attachment	Agar plates and top agar for plaque formation	Agar plates for excision protocol
XLOLR strain	LB-tetracycline[a]	LB broth with supplements[a-c]	—	LB-kanamycin[a]
VCS257 strain[d]	LB[a]	LB broth with supplements[a-c]	NZY[a]	—
XL1-Blue MRF′ strain	LB-tetracycline[a]	LB broth with supplements[a-c]	NZY[a]	—

[a] See *Preparation of Media and Reagents*.

[b] LB broth with 0.2% (w/v) maltose and 10 mM $MgSO_4$.

[c] Maltose and magnesium supplements are required for optimal lambda phage receptor expression on the surface of the XL1-Blue MRF′ host cell. The media supplements are not required for helper phage infection, but are included in both protocols for simplified media preparation.

[d] For use with Gigapack III Gold packaging extract and wild-type control only. Supplied with Gigapack III Gold packaging extract.

Establishing an Agar Plate Bacterial Stock

The bacterial host strains are shipped as bacterial glycerol stocks. On arrival, prepare the following plates from the bacterial glycerol stocks.

Note *The host strains may thaw during shipment. The vials should be stored immediately at –20° or –80°C, but most strains remain viable longer if stored at –80°C. It is best to avoid repeated thawing of the host strains in order to maintain extended viability.*

1. Revive the stored cells by scraping off splinters of solid ice with a sterile wire loop.
2. Streak the splinters onto an LB agar plate containing the appropriate antibiotic (see *Recommended Media*), if one is necessary.
3. Incubate the plate overnight at 37°C.
4. Seal the plate with Parafilm® laboratory film and store the plate at 4°C for up to 1 week.
5. Restreak the cells onto a fresh plate every week.

Preparing a –80°C Bacterial Glycerol Stock

1. In a sterile 50-ml conical tube, inoculate 10 ml of LB broth with the appropriate antibiotic (see *Recommended Media*) with one colony from the plate. Grow the cells to late log phase.

2. Add 4.5 ml of a sterile glycerol-liquid medium solution (prepared by mixing 5 ml of glycerol + 5 ml of the appropriate medium) to the bacterial culture from step 1. Mix well.

3. Aliquot into sterile centrifuge tubes (1 ml/tube).

This preparation may be stored at –20°C for 1–2 years or at –80°C for more than 2 years.

Color Selection by IPTG and X-gal

The color selection by α-complementation with the ZAP Express vector requires a high amount of IPTG and X-gal for generation of the blue color (see *Plating and Titering*). Transcription and translation of the fusion protein are normal, but the large polylinker present within the pBK-CMV phagemid vector, which is present in the ZAP Express vector, is partly responsible for the reduced activity of the β-galactosidase protein—not the promoter. As would be expected, the copy number of the ZAP Express vector is much less per cell than the copy number of pBK-CMV phagemid vector derivatives. However, it is important to note that the color assay is used only for determining the ratio of recombinants to nonrecombinants within a newly constructed library and is not used for any other manipulations.

Growth of Cells for Plating Phage

Bacterial cultures for plating phage should be started from a fresh plate using a single colony and should be grown overnight with vigorous shaking at 30°C in 50 ml of LB broth supplemented with 0.2% (w/v) maltose and 10 mM $MgSO_4$. (Do not use tetracycline in the presence of magnesium.) The lower temperature ensures that the cells will not overgrow. The cells should be spun at 1000 × *g* for 10 minutes then gently resuspended in 10 ml of 10 mM $MgSO_4$. Before use, dilute cells to an OD_{600} of 0.5 with 10 mM $MgSO_4$. Bacterial cells prepared in this manner can be used for all phage manipulations described within the manual. Highest efficiencies are obtained from freshly prepared cells.

HELPER PHAGE

Two different helper phages are provided with the ZAP Express cDNA synthesis kit: (1) the ExAssist interference-resistant helper phage with XLOLR strain and (2) the R408 helper phage. The ExAssist interference-resistant helper phage with XLOLR strain is designed to allow efficient in vivo excision of the pBK-CMV phagemid vector from the ZAP Express vector while preventing problems associated with helper phage co-infection. The ExAssist helper phage contains an amber mutation that prevents replication of the phage genome in a nonsuppressing *E. coli* strain (e.g., XLOLR cells). Only the excised phagemid can replicate in the host, removing the possibility of co-infection from the ExAssist helper phage. Because ExAssist helper phage cannot replicate in the XLOLR strain, single-stranded rescue cannot be performed in this strain using ExAssist helper phage. XLOLR cells are also resistant to lambda infection, preventing lambda DNA contamination after excision.

Storing the Helper Phage

The ExAssist helper phage and the R408 helper phage are supplied in 7% dimethylsulfoxide (DMSO) and should be stored at –80°C. The helper phage may be stored for short periods of time at –20°C or 4°C. It is important to titer the helper phage prior to each use. Expect titers of approximately 10^{10} pfu/ml for the ExAssist helper phage or 10^{10} pfu/ml for the R408 helper phage. If the titer drops over time, prepare a fresh high-titer stock of the helper phage as outlined in *Amplifying the Helper Phage*.

Titering the Helper Phage

1. Transfer a colony of XL1-Blue MRF´ cells into 10 ml of LB broth with supplements in a 50-ml conical tube. Incubate the conical tube with shaking at 37°C until growth reaches an OD_{600} of 1.0.

2. Dilute the phage (10^{-4}–10^{-7}) in SM buffer (See *Preparation of Media and Reagents*) and combine 1 µl of each dilution with 200 µl of XL1-Blue MRF´ cells (OD_{600} = 1.0).

3. Incubate the helper phage and the XL1-Blue MRF´ cells for 15 minutes at 37°C to allow the phage to attach to the cells.

4. Add 3 ml of NZY top agar, melted and cooled to ~48°C, and plate immediately onto dry, prewarmed NZY agar plates. Allow the plates to set for 10 minutes.

5. Invert the plates and incubate overnight at 37°C.

   Note *ExAssist and R408 plaques will have a cloudier appearance than lambda phage plaques.*

6. To determine the titer [in plaque-forming units per milliliter (pfu/ml)], use the following formula:

$$\left[\frac{\text{Number of plaques (pfu)} \times \text{dilution factor}}{\text{Volume plated (µl)}}\right] \times 1000 \text{ µl / ml}$$

   where the volume plated (in microliters) refers to the volume of the helper phage solution added to the cells.

Amplifying the Helper Phage

1. Transfer a colony of XL1-Blue MRF′ cells into 10 ml of LB broth with supplements in a 50-ml conical tube. Incubate the conical tube with shaking at 37°C until growth reaches an OD_{600} of 0.3.

   Note *An OD_{600} of 0.3 corresponds to 2.5 × 10^8 cells/ml.*

2. Add the helper phage at a multiplicity of infection (MOI) of 20:1 (phage-to-cells ratio).

3. Incubate the conical tube at 37°C for 15 minutes to allow the phage to attach to the cells.

4. Incubate the conical tube with shaking at 37°C for 8 hours.

5. Heat the conical tube at 65°C for 15 minutes.

6. Spin down the cell debris and transfer the supernatant to a fresh conical tube.

7. The titer of the supernatant should be between 7.5 × 10^{10} and 1.0 × 10^{12} pfu/ml for ExAssist helper phage or between 1.0 × 10^{11} and 1.0 × 10^{12} pfu/ml for R408 helper phage.

   Note *ExAssist and R408 plaques will have a cloudier appearance than lambda phage plaques.*

8. Add dimethylsulfoxide (DMSO) to a final concentration of 7% (v/v) and store at –80°C.

9. For further details about helper phage titering or amplification, please see *Titering the Helper Phage* or Reference 6.

PACKAGING EXTRACTS

Packaging extracts are used to package recombinant lambda phage with high efficiency, which increases the size of gene libraries.

Gigapack III Gold packaging extract increases the efficiency and representation of libraries constructed from highly methylated DNA. The packaging extracts are restriction minus ($HsdR^-$ $McrA^-$ $McrBC^-$ $McrF^-$ Mrr^-) to optimize packaging efficiency and library representation. When used in conjunction with restriction-deficient plating cultures, Gigapack III Gold packaging extract should improve the quality of DNA libraries constructed from methylated DNA.[2,79]

Optimal packaging efficiencies are obtained with lambda DNAs that are concatemeric. Ligations should be carried out at DNA concentrations of 0.2 µg/ µl or greater, which favors concatemers and not circular DNA molecules that only contain one *cos* site. DNA to be packaged should be relatively free from contaminants. DNA may be used directly from ligation reactions in most cases; however, polyethylene glycol (PEG), which is contained in some ligase buffers, has been shown to inhibit packaging. The volume of DNA added to each extract should be <5 µl.

Undigested wild-type lambda DNA will be packaged with efficiencies exceeding 1×10^9 plaques/µg of vector when using Gigapack III Gold packaging extract. Predigested arms, when ligated to a test insert, will yield ~5×10^6 recombinant plaques/µg of vector.

THE ZAP EXPRESS cDNA SYNTHESIS PROTOCOL

Notes ***DO NOT*** *substitute the reagents listed below with reagents from another kit. Component substitution may result in lower efficiency library construction.*

The following protocol uses radiolabeled nucleotides in control first- and second-strand synthesis reactions to assess the quality and the size of the cDNA synthesis products. An alternative protocol, using SYBR Green II staining instead of ^{32}P *labeling, is available in a Technical Note on the Stratagene website:* http://www.stratagene.com/lit_items/cDNA_Synthesis_Kit-Using_SYBR®.pdf.

The following protocol is optimized for 5 µg of poly(A)⁺ RNA.

Protocol Guidelines

- The quality and quantity of the mRNA used is of fundamental importance to the construction of a large, representative cDNA library (see *Appendix I: Purifying and Quantifying RNA*). The Stratagene RNA Isolation Kit (Catalog #200345) uses the guanidinium thiocyanate–phenol–chloroform extraction method,[10] which quickly produces large amounts of undegraded RNA. To isolate mRNA, the Stratagene Products Division offers the Poly(A) Quik mRNA isolation kit [Catalog #200348 (2 columns) and #200349 (4 columns)].

- Secondary structure of mRNA may cause the synthesis of truncated cDNAs. To relax secondary structure, treatment with methylmercury hydroxide (CH_3HgOH) is recommended (see *Appendix II: Treating with Methylmercury Hydroxide*).

- It is imperative to protect the RNA from any contaminating RNases until the first-strand cDNA synthesis is complete. Wear fresh gloves, use newly autoclaved pipet tips, and avoid using pipet tips or microcentrifuge tubes that have been handled without gloves. Ribonuclease A *cannot* be destroyed by normal autoclaving alone. Baking or DEPC treatment is recommended.

- When removing aliquots of any of the enzymes used in the ZAP Express cDNA synthesis protocol, flick the bottom of the tube to thoroughly mix the enzyme solution. Do not vortex the enzyme stock tubes.

Synthesizing First-Strand cDNA

1. Preheat a 42°C water bath.

2. Thaw the radioactive [α-^{32}P]dNTP (do not use [^{32}P]dCTP) and all nonenzymatic first-strand components. Keep the radioactive dNTP on ice for use in step 6.and in the second-strand synthesis. Briefly vortex and spin down the contents of the nonenzymatic tubes. Place the tubes on ice.

   Note *AccuScript RT is temperature sensitive and should remain at –20°C until the last moment.*

3. The final volume of the first-strand synthesis reaction is 50 µl. The volume of added reagents and enzymes is 14 µl, thus the mRNA template and DEPC-treated water should be added in a combined volume of 36 µl. For the control reaction, prepare the following annealing reaction with 25 µl (5 µg) of test RNA and 11 µl of DEPC-treated water.

4. In an RNase-free microcentrifuge tube, add the following reagents in order:

   5 µl of 10× first-strand buffer
   3 µl of first-strand methyl nucleotide mixture
   2 µl of linker–primer (1.4 µg/µl)
   X µl of DEPC-treated water
   1 µl of RNase Block Ribonuclease Inhibitor (40 U/µl)

5. Mix the reaction and then add *X* µl of poly(A)$^+$ RNA (5 µg). Mix gently.

6. Allow the primer to anneal to the template for 10 minutes at room temperature. During the incubation, aliquot 0.5 µl of the [α-^{32}P]dNTP (800 Ci/mmol) into a separate tube for the control.

7. Add 3 µl of AccuScript RT to the first-strand synthesis reaction. The final volume of the first-strand synthesis reaction should now be 50 µl.

8. Mix the sample gently and spin down the contents in a microcentrifuge.

9. **Transfer 5 µl of the first-strand synthesis reaction to the separate tube containing the 0.5 µl of the [α-^{32}P]dNTP (800 Ci/mmol). This radioactive sample is the first-strand synthesis control reaction.**

10. Incubate the first-strand synthesis reactions, including the control reaction, at 42°C for 1 hour.

11. Prepare a 16°C water bath for second-strand synthesis. If a water bath with a cooling unit is not available, use a large Styrofoam® container with a lid. Fill the container three-quarters full with water and adjust the temperature to 16°C with ice. Cover the container with a lid.

12. After 1 hour, remove the first-strand synthesis reactions from the 42°C water bath. Place the nonradioactive first-strand synthesis reaction on ice. Store the radioactive first-strand synthesis control reaction at –20°C until ready to resolve by electrophoresis on an alkaline agarose gel (see *Appendix III: Alkaline Agarose Gels*). It may be helpful to run the radioactive first-strand reaction after the second-strand reaction has been blunted and resuspended in the *Eco*R I adapters (see step 17 in *Blunting the cDNA Termini*).

Synthesizing Second-Strand cDNA

1. Thaw all nonenzymatic second-strand components. Briefly vortex and spin in a micro-centrifuge before placing the tubes on ice.

   Note *It is important that all reagents be <16°C when the DNA polymerase I is added.*

2. Add the following components in order to the 45-µl nonradioactive, first-strand synthesis reaction on ice:

   20 µl of 10× second-strand buffer
   6 µl of second-strand dNTP mixture
   114 µl of sterile distilled water (DEPC-treated water is not required)
   2 µl of [α-^{32}P]dNTP (800 Ci/mmol)

3. Add the following enzymes to the second-strand synthesis reaction:

   2 µl of RNase H (1.5 U/µl)
   11 µl of DNA polymerase I (9.0 U/µl)

4. Gently vortex the contents of the tube, spin the reaction in a microcentrifuge, and incubate for 2.5 hours at 16°C. Check the water bath occasionally to ensure that the temperature does not rise above 16°C. Temperatures above 16°C can cause the formation of hairpin structures, which are unclonable and interfere with the efficient insertion of correctly synthesized cDNA into the prepared vector.

5. After second-strand synthesis for 2.5 hours at 16°C, immediately place the tube *on ice.*

Blunting the cDNA Termini

1. Add the following to the second-strand synthesis reaction:

   23 µl of blunting dNTP mix
   2 µl of cloned *Pfu* DNA polymerase (2.5 U/µl)

2. Quickly vortex the reaction and spin in a microcentrifuge. Incubate the reaction at 72°C for 30 minutes. **Do not exceed 30 minutes!!**

3. Thaw the 3 M sodium acetate.

   Note *Since radioactivity can leak out between the lid and body of some micro-centrifuge tubes during the vortexing and precipitation steps, wrap a small piece of Parafilm laboratory film around the rim of the microcentrifuge tube to prevent leakage.*

4. Remove the reaction and add 200 µl of phenol–chloroform [1:1 (v/v)] and vortex.

   Note ***Do not use the low-pH phenol from the Stratagene RNA Isolation Kit because this phenol is acidic and may denature the DNA.*** *The phenol must be equilibrated to pH of 7–8.*

5. Spin the reaction in a microcentrifuge at maximum speed for 2 minutes at room temperature and transfer the upper aqueous layer, containing the cDNA, to a new tube. Be careful to avoid removing any interface that may be present.

6. Add an equal volume of chloroform and vortex.

7. Spin the reaction in a microcentrifuge at maximum speed for 2 minutes at room temperature and transfer the upper aqueous layer, containing the cDNA, to a new tube.

8. Precipitate the cDNA by adding the following to the saved aqueous layer:

   20 µl of 3 M sodium acetate
   400 µl of 100% (v/v) ethanol

   Vortex the reaction.

9. Precipitate overnight at –20°C.

10. In order to orient the direction of precipitate accumulation, place a mark on the microcentrifuge tube or point the tube hinge away from the center of the microcentrifuge as an indicator of where the pellet will form.

11. Spin in a microcentrifuge at maximum speed for 60 minutes at 4°C.

12. Avoid disturbing the pellet and carefully remove and discard the radioactive supernatant in a radioactive waste container.

    Note *The conditions of synthesis and precipitation produce a large white pellet. The pellet accumulates near the bottom of the microcentrifuge tube and may taper up along the marked side of the tube.*

13. Gently wash the pellet by adding 500 µl of 70% (v/v) ethanol to the side of the tube away from the precipitate. **Do not mix or vortex!**

14. Spin in a microcentrifuge at maximum speed for 2 minutes at room temperature with the orientation marked as in step 10.

15. Aspirate the ethanol wash and dry the pellet by vacuum centrifugation.

16. Resuspend the pellet in 9 µl of *Eco*R I adapters and incubate at 4°C for at least 30 minutes to allow the cDNA to resuspend. To ensure that the cDNA is completely in solution, transfer the cDNA to a fresh microcentrifuge tube. Monitor the now empty tube with a handheld Geiger counter. If the cDNA is in solution, few counts should remain in the empty tube.

17. **Transfer 1 µl of this second-strand synthesis reaction to a separate tube. This radioactive sample is the second-strand synthesis control reaction.** At this point, run the samples of the first- and second-strand synthesis reaction on an alkaline agarose gel. It is important to determine the size range of the cDNA and the presence of any secondary structure (see *Appendix III: Alkaline Agarose Gels*).

    Note *The second-strand synthesis reaction can be stored overnight at –20°C.*

Ligating the *Eco*R I Adapters

1. Add the following components to the tube containing the blunted cDNA and the *Eco*R I adapters:

   1 µl of 10× ligase buffer
   1 µl of 10 mM rATP
   1 µl of T4 DNA ligase (4 U/µl)

2. Spin down the volume in a microcentrifuge and incubate overnight at 8°C. Alternatively, the ligations can be incubated at 4°C for 2 days.

3. In the morning, heat inactivate the ligase by placing the tubes in a 70°C water bath for 30 minutes.

Phosphorylating the *Eco*R I Ends

1. After the ligase is heat inactivated, spin the reaction in a microcentrifuge for 2 seconds. Cool the reaction at room temperature for 5 minutes.

2. Phosphorylate the adapter ends by adding the following components:

   1 µl of 10× ligase buffer
   2 µl of 10 mM rATP
   5 µl of sterile water
   2 µl of T4 polynucleotide kinase (5 U/µl)

3. Incubate the reaction for 30 minutes at 37°C.

4. Heat inactivate the kinase for 30 minutes at 70°C.

5. Spin down the condensation in a microcentrifuge for 2 seconds and allow the reaction to equilibrate to room temperature for 5 minutes.

Digesting with *Xho* I

1. Add the following components to the reaction:

   28 µl of *Xho* I buffer supplement
   3 µl of *Xho* I (40 U/µl)

2. Incubate the reaction for 1.5 hours at 37°C.

3. Add 5 µl of 10× STE buffer and 125 µl of 100% (v/v) ethanol to the microcentrifuge tube.

4. Precipitate the reaction overnight at –20°C.

5. Following precipitation, spin the reaction in a microcentrifuge at maximum speed for 60 minutes at 4°C.

6. Discard the supernatant, dry the pellet completely, and resuspend the pellet in 14 µl of 1× STE buffer.

7. Add 3.5 µl of the column loading dye to each sample.

The sample is now ready to be run through a drip column containing Sepharose CL-2B gel filtration medium (see *Size Fractionating*).

Size Fractionating

Before attempting the experimental protocols outlined within this section, please read this section in its entirety in order to become familiar with the procedures. Review of the *Troubleshooting* section may also prove helpful. The drip columns should be prepared and the cDNA should be eluted in 1 day. Because a full day is required to complete these procedures, gathering all necessary materials in advance is recommended (see the *Equipment* section in *Additional Materials Required*).

Assembling the Drip Column

1. Perform the following preparatory steps while assembling the drip columns:

    a. Remove the Sepharose CL-2B gel filtration medium and the 10× STE buffer from refrigeration and equilibrate the two components to room temperature.

    b. Prepare 50 ml of 1× STE buffer by diluting 10× STE buffer 1:10 in sterile water.

2. Assemble the drip columns as outlined in the following steps (see Figure 4 for a diagram of the final setup):

    Note *Wear gloves while assembling the drip columns.*

    a. Remove the plastic wrapper from the top of a sterile 1-ml pipet.

    b. Using a sterile needle or a pair of fine-tipped forceps, **carefully** tease the cotton plug out of each pipet, leaving a piece of the cotton plug measuring ~3–4 mm inside. Cut off the external portion of the cotton plug.

    c. Push the remaining 3- to 4-mm piece of the cotton plug into the top of each pipet with the tip of the needle or forceps.

    d. Cut a small piece of the connecting tubing measuring ~8 mm. Use this small tube to connect the 1-ml pipet to the 10-ml syringe. First attach one end of the connecting tube to the pipet and then connect the other end to the syringe. There should be no gap between the pipet and the syringe when joined by the connecting tube.

        Note *The inside diameter of the connecting tubing (~1/8-inch i.d.) snugly connects most disposable 1-ml pipets and the ends of all B-D® 10-cc syringes with the Luer Lok® tips.*

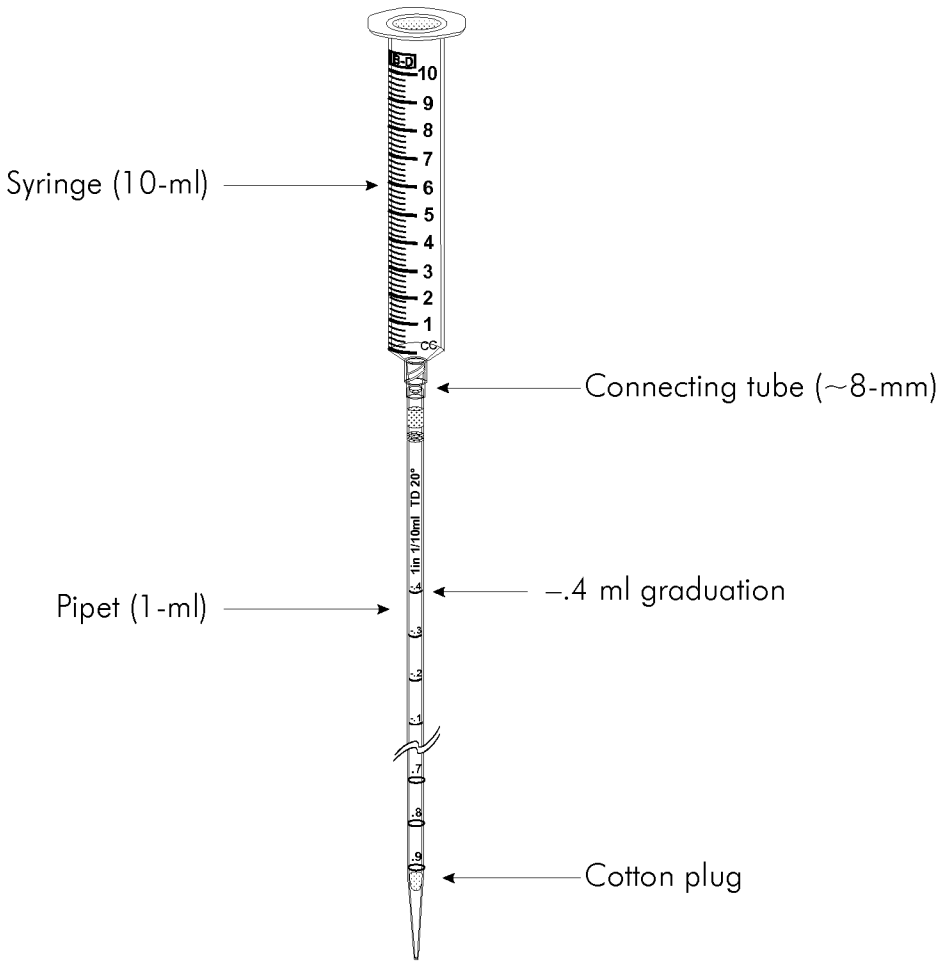


FIGURE 4 Assembly of the drip columns.

e. Rapidly and forcefully push the plunger into the syringe to thrust the cotton plug down into the tip of the pipet.

> ***Note*** *It may take several attempts to drive the cotton all the way down into the tip of the pipet. However, pushing the cotton plug as far down into the pipet tip as possible is important in order to achieve optimal separation of the cDNA fractions.*

f. Remove the plunger from the syringe. Because the syringe functions as a buffer reservoir for the drip column, leave the syringe firmly attached to the pipet throughout the remainder of the size fractionation procedure.

3. Locate a support for the assembled drip column. Butterfly clamps or a three-fingered clamp on a ring stand can be used.

Loading the Drip Column

1. Load the drip column with a uniform suspension of Sepharose CL-2B gel filtration medium as outlined in the following steps:

   a. Immediately prior to loading the drip column, gently mix the Sepharose CL-2B gel filtration medium by inversion until the resin is uniformly suspended.

   b. Place the column in the ring stand. Fill a glass Pasteur pipet with ~2 ml of 1× STE buffer. Insert the pipet as far into the drip column as possible and fill the column with the buffer.

      Notes *If the 1× STE buffer flows too quickly through the column, stem the flow by affixing a yellow pipet tip to the end of the column. Make sure to remove the pipet tip prior to loading the column with the Sepharose CL-2B gel filtration medium.*

      If bubbles or pockets of air become trapped in the STE buffer while filling the column, remove the trapped air prior to packing the column with the resin. To remove the bubbles or air, re-insert the Pasteur pipet into the top of the column and gently pipet the STE buffer in and out of the pipet until the trapped air escapes through the top of the column.

   c. Immediately add a uniform suspension of Sepharose CL-2B gel filtration medium to the column with a Pasteur pipet by inserting the pipet as far into the column as possible. As the resin settles, continue adding the Sepharose CL-2B gel filtration medium. Stop adding the resin when the surface of the packed bed is ¼ inch below the "lip of the pipet." The lip of the pipet is defined as the point where the pipet and the syringe are joined.

      Notes *If air bubbles form as the resin packs, use a Pasteur pipet as described in step 1b to remove the blockage. Failure to remove bubbles can impede the flow of the column and result in a loss of the cDNA.*

      If the preparation of Sepharose CL-2B gel filtration medium settles and becomes too viscous to transfer from the stock tube to the column, add a small volume (~1–5 ml) of 1× STE buffer to resuspend the resin.

2. Wash the drip column by filling the buffer reservoir (i.e., the syringe) with a minimum of 10 ml of 1× STE buffer. As the column washes, the buffer should flow through the drip column at a steady rate; however, it may take at least 2 hours to complete the entire wash step. After washing, do not allow the drip column to dry out, because the resin could be damaged and cause sample loss. If this occurs, pour another column.

   Note *If a free flow of buffer is not observed, then bubbles or pockets of air have become trapped in the drip column. In this case, the column must be repacked. If cDNA is loaded onto a column on which a free flow of buffer is not observed, the sample could become irretrievably lost.*

3. When ~50 µl of the STE buffer remains above the surface of the resin, immediately load the cDNA sample using a pipettor. Gently release the sample onto the surface of the column bed, but avoid disturbing the resin as this may affect cDNA separation.

4. Once the sample enters the Sepharose CL-2B gel filtration medium, fill the connecting tube with buffer using a pipettor.

   Note *Do not disturb the bed while filling the connecting tube with buffer.*

   Gently add 3 ml of 1× STE buffer to the buffer reservoir by trickling the buffer down the inside wall of the syringe. Do not squirt the buffer into the reservoir because this will disturb the resin, resulting in loss of the sample.

5. As the cDNA sample elutes through the column, the dye will gradually diffuse as it migrates through the resin. Because the dye is used to gauge when the sample elutes from the column, monitor the progress of the dye, or the cDNA sample could be irretrievably lost.

Collecting the Sample Fractions

The drip column containing the Sepharose CL-2B gel filtration medium separates molecules on the basis of size. Large cDNA molecules elute first followed by smaller cDNA and finally unincorporated nucleotides. Using a handheld monitor, two peaks of radioactivity can generally be detected during the course of elution. The first peak to elute from the column represents the cDNA. Due to the conditions of label incorporation during second-strand synthesis, the cDNA is not extremely radioactive; therefore, the counts per second may be barely above background levels. In contrast, the second peak to elute from the column is highly radioactive as this is the unincorporated radioactive nucleotides. Although this material elutes from the column in parallel with the dye, unincorporated nucleotides are usually not collected because the cDNA has already eluted from the column.

For standard cDNA size fractionation (>400 bp), collect ~12 fractions using the procedure described in this section. The progression of the leading edge of the dye through the column will be used as a guideline to monitor collection; however, the drops collected from the column should be monitored for radioactivity using a handheld Geiger counter. Until the fractions have been assessed for the presence of cDNA on a 5% nondenaturing acrylamide gel (see *Preparation of Media and Reagents*), do not discard any fractions based on the quantity of radioactivity detected.

1. Using a fresh microcentrifuge tube to collect each fraction, begin collecting **three drops** per fraction when the leading edge of the dye reaches the **–0.4-ml** gradation on the pipet.

2. Continue to collect fractions until the trailing edge of the dye reaches the **0.3-ml** gradation. A minimum of 12 fractions, each containing ~100 µl (i.e., three drops), should be collected. Alternatively, fractions can be collected until the radioactive-free nucleotides begin to elute. In either case, monitor the fractions for the presence of radioactivity to determine whether the cDNA has eluted successfully. If no counts are detected, continue collecting the fractions until the peak of unincorporated nucleotides is recovered.

3. Before processing the fractions and recovering the size-fractionated cDNA, **remove 8 µl of each collected fraction and save for later analysis. These aliquots will be electrophoresed on a 5% nondenaturing acrylamide gel** to assess the effectiveness of the size fractionation and to determine which fractions will be used for ligation.

Processing the cDNA Fractions

In this section of the size fractionation procedure, the fractions collected from the drip column are extracted with phenol–chloroform and are precipitated with ethanol to recover the size-selected cDNA. The purpose of the organic extractions is to remove contaminating proteins; of particular concern is kinase, which can be carried over from previous steps in the synthesis. Because kinase often retains activity following heat treatment, it is necessary to follow the extraction procedures.

1. Begin extracting the remainder of the collected fractions by adding an equal volume of phenol–chloroform [1:1 (v/v)].

2. Vortex and spin in a microcentrifuge at maximum speed for 2 minutes at room temperature. Transfer the upper aqueous layer to a fresh microcentrifuge tube.

3. Add an equal volume of chloroform.

4. Vortex and spin in a microcentrifuge at maximum speed for 2 minutes at room temperature. Transfer the upper aqueous layer to a fresh microcentrifuge tube.

5. To each extracted sample, add a volume of 100% (v/v) ethanol that is equal to twice the individual sample volume.

   Note *The 1× STE buffer contains sufficient NaCl for precipitation.*

6. Precipitate overnight at –20°C.

7. Spin the sample in the microcentrifuge at maximum speed for 60 minutes at 4°C. Transfer the supernatant to another tube. To ensure that the cDNA has been recovered, use a handheld Geiger counter to check the level of radioactivity present in the pellet. If the majority of the radiation is detected in the supernatant, repeat the centrifugation step; otherwise, discard the supernatant.

8. Carefully wash the pellet with 200 µl of 80% (v/v) ethanol, ensuring that the pellet remains undisturbed. *Do not mix or vortex!* Spin the sample in a microcentrifuge at maximum speed for 2 minutes at room temperature. Remove the ethanol and verify that the pellet has been recovered by visual inspection or with the handheld Geiger counter. Vacuum evaporate the pellet for ~5 minutes or until dry. Do not dry the pellet beyond the point of initial dryness or the cDNA may be difficult to solubilize.

9. Using a handheld Geiger counter verify that the cDNA has been recovered and record the number of counts per second (cps) that is detected for each fraction.

10. If <30 cps is detected, resuspend each cDNA pellet in 3.5 µl of sterile water. If the value is >30 cps, resuspend the cDNA in 5 µl of sterile water. Mix by pipetting up and down.

To help ensure ligation success, quantitate the cDNA before proceeding (see *Appendix IV: Ethidium Bromide Plate Assay—Quantitating the cDNA*). Best results are usually obtained by ligating 100 ng of cDNA/1 µg of vector. Place the remaining cDNA at –20°C for short term storage only. The cDNA is most stable after ligation into vector arms and may be damaged during long-term storage.

Ligating cDNA into the ZAP Express Vector

Important ***Use the ligase buffer provided with the cDNA Synthesis Kit.*** *Polyethylene glycol, which is present in some ligase buffers, can inhibit packaging.*

1. Set up a control ligation to ligate the test insert into the ZAP Express vector as follows:

   1.0 µl of the ZAP Express vector (1 µg)
   1.6 µl of test insert (0.4 µg)
   0.5 µl of 10× ligase buffer
   0.5 µl of 10 mM rATP (pH 7.5)
   0.9 µl of water

   Then add 0.5 µl of T4 DNA ligase (4 U/µl)

2. To prepare the sample ligation, add the following components:

   X µl of resuspended cDNA (~100 ng)
   0.5 µl of 10× ligase buffer
   0.5 µl of 10 mM rATP (pH 7.5)
   1.0 µl of the ZAP Express vector (1 µg/µl)
   X µl of water for a final volume of 4.5 µl

   Then add 0.5 µl of T4 DNA ligase (4 U/µl)

3. Incubate the reaction tubes overnight at 12°C or for up to 2 days at 4°C.

4. If the library is to be packaged the following day, start a 50-ml culture of XL1-Blue MRF´ cells from a colony isolated on a tetracycline agar plate. At the same time, start a 50-ml culture of VCS257 cells for plating the wild-type lambda control DNA used to test the Gigapack III Gold packaging extract. See the table in *Bacterial Host Strains* for appropriate growth media.

   Note *XL1-Blue MRF´ cells are RecA$^-$ and consequently grow slowly. See the table in* Bacterial Host Strains *for detailed explanations of optimal media.*

5. After ligation is complete, package 1 µl of each ligation, including the control ligation, using Gigapack III Gold packaging extract according to the packaging instructions outlined in *Packaging*. A good representational primary library size consists of ~1 × 10^6 clones. If a low number of plaque-forming units results from packaging the 1-µl ligation, try packaging 2–3 µl of the remaining ligation mixture in one packaging reaction.

   Note *Use high-efficiency Gigapack III Gold packaging extract since this packaging extract is McrA$^-$, McrB$^-$, and Mrr$^-$.*[2] *Other commercially available packaging extracts can restrict hemimethylated DNA, therefore producing low-titer libraries.*

PACKAGING

Preparing the Host Bacteria

Note *The VCS257 strain is for use with the Gigapack III Gold packaging extract and the positive wild-type lambda DNA control only.*

1. Streak the XL1-Blue MRF´ and VCS257 cells onto LB agar plates containing the appropriate antibiotic (See *Recommended Media*). Incubate the plates overnight at 37°C.

   Note *Do not add antibiotic to the medium in the following step. The antibiotic will bind to the bacterial cell wall and will inhibit the ability of the phage to infect the cell.*

2. Prepare separate 50-ml cultures of XL1-Blue MRF´ and VCS257 cells in LB broth with supplements.

3. Incubate with shaking at 37°C for 4–6 hours (do not grow past an OD_{600} of 1.0). Alternatively, grow overnight at 30°C, shaking at 200 rpm.

   Note *The lower temperature keeps the bacteria from overgrowing, thus reducing the number of nonviable cells. Phage can adhere to nonviable cells resulting in a decreased titer.*

4. Pellet the bacteria at 1000 × *g* for 10 minutes.

5. Gently resuspend each cell pellet in 25 ml sterile 10 mM $MgSO_4$.

   Note *For later use, store the cells at 4°C overnight in 10 mM $MgSO_4$.*

6. Dilute the cells to an OD_{600} of 0.5 with sterile 10 mM $MgSO_4$.

   Note *The bacteria should be used immediately following dilution.*

7. If using Gigapack III Gold packaging extract, follow the instructions outlined in *Packaging Protocol for Gigapack III Gold Packaging Extract* and *Testing the Efficiency of the Gigapack III Gold Packaging Extract with the Wild-Type Lambda Control DNA (Optional).*

Packaging Protocol for the Gigapack III Gold Packaging Extract

Important ***Use the ligase buffer provided with the ZAP Express cDNA synthesis kit.*** *Polyethylene glycol, which is contained in some ligase buffers, can inhibit packaging.*

1. Remove the appropriate number of packaging extracts from the –80°C freezer and place the extracts on dry ice.

2. Quickly thaw the packaging extract by holding the tube between your fingers until the contents of the tube just begins to thaw.

3. Add the experimental DNA **immediately** (1–4 µl containing 0.1–1.0 µg of ligated DNA) to the packaging extract.

4. Stir the reaction mixture with a pipet tip to mix well. **Gentle** pipetting is allowable provided that air bubbles are not introduced.

5. Spin the tube quickly (for 3–5 seconds), if desired, to ensure that all contents are at the bottom of the tube.

6. Incubate the tube at room temperature (22°C) for 2 hours. **Do not exceed 2 hours.**

   Note *The highest efficiency occurs between 90 minutes and 2 hours. Efficiency may drop dramatically during extended packaging times.*

7. Add 500 µl of SM buffer to the tube.

8. Add 20 µl of chloroform and mix the contents of the tube gently.

9. Spin the tube briefly to sediment the debris.

10. The supernatant containing the phage is now ready to be titered and may be stored at 4°C.

Testing the Efficiency of the Gigapack III Gold Packaging Extract with the Wild-Type Lambda Control DNA (Optional)

Use the following procedure to test the efficiency of Gigapack III Gold packaging extract with the λ*c*I857 *Sam*7 wild-type lambda control DNA.

1. Thaw the frozen wild-type lambda control DNA on ice and gently mix the control after thawing.

2. Using 1 µl of the wild-type lambda control DNA (~0.2 µg), proceed with steps 1–10 in the *Packaging Protocol for the Gigapack III Gold Packaging Extract.*

   Note *Because of the high titer achieved with the wild-type lambda control DNA, stop the control packaging reaction with 1 ml of SM buffer. This should make the plaques easier to count.*

3. Prepare two consecutive 10^{-2} dilutions of the packaging reaction from step 10 in the *Packaging Protocol for the Gigapack III Gold Packaging Extract* in SM buffer. (The final dilution is 10^{-4}).

4. Add 10 µl of the 10^{-4} dilution to 200 µl of the VCS257 host strain at an OD_{600} of 0.5. (This strain is recommended for plating the wild-type lambda control DNA only.) Incubate at 37°C for 15 minutes. Add 3 ml of NZY top agar, melted and cooled to ~48°C, and quickly pour the dilution onto dry, prewarmed NZY agar plates.

5. Incubate the plates for at least 12 hours at 37°C.

6. Count the plaques. Approximately 400 plaques should be obtained on the 10^{-4} dilution plate with Gigapack III Gold packaging extract.

7. Calculate the efficiency using the following equation:

$$\frac{\text{Number of plaques} \times \text{dilution factor} \times \text{total packaging volume}}{\text{Total number of micrograms packaged} \times \text{number of microliters plated}}$$

PLATING AND TITERING

A background test can be completed by plating several hundred plaques on an indicator plate (see *Color Selection by IPTG and X-gal*). Add 15 µl of 0.5 M IPTG (in water) and 50 µl of 250 mg/ml X-gal [in dimethylformamide (DMF)] to 2–3 ml of NZY top agar, melted and cooled to ~48°C. The high concentrations of IPTG and X-gal used for plating often result in the formation of a precipitate, which disappears after incubation. To minimize the formation of this precipitate, the IPTG and X-gal should be added separately to the NZY top agar, with mixing in between additions. Plate immediately on NZY agar plates. Plaques are visible after incubation for 6–8 hours at 37°C. Background plaques are blue, while recombinant plaques are white.

1. Prepare the host bacteria as outlined in *Preparing the Host Bacteria.*

2. Dilute the lambda phage in SM buffer as follows:

   For primary library titering, add 1 µl of the lambda phage to 200 µl of host cells diluted in 10 mM $MgSO_4$ to OD_{600} = 0.5. If desired, also add 1 µl of a 1:10 dilution of the packaged material in SM buffer to 200 µl of host cells.

   For amplified library titering, first dilute the amplified phage stock in SM buffer by the following amounts: 1:10,000, 1:100,000, 1:1,000,000. Add 1 µl of each dilution to 200 µl of host cells.

3. Incubate the phage and bacteria for 15 minutes at 37°C to allow the phage to attach to the cells.

4. Add the following components:

   2–3 ml of NZY top agar (melted and cooled to ~48°C).
   15 µl of 0.5M IPTG (in water)
   50 µl of X-gal [250 mg/ml (in DMF)]

5. Plate immediately onto dry, prewarmed NZY agar plates and allow the plates to set for 10 minutes. Invert the plates and incubate at 37°C.

6. Plaques should be visible after 6–8 hours, although color detection requires overnight incubation. Background plaques are blue and should be $< 1 \times 10^5$ pfu/µg of arms, while recombinant plaques will be white (clear) and should be 10–100-fold above the background

   Note *Primary libraries can be unstable; therefore, amplification of the libraries is recommended immediately.*

AMPLIFYING THE ZAP EXPRESS LIBRARY

It is usually desirable to amplify libraries prepared in lambda vectors to make a large, stable quantity of a high-titer stock of the library. However, more than one round of amplification is not recommended, since slower growing clones may be significantly underrepresented. The following protocol is recommended for amplifying the ZAP Express library.

Day 1

1. Grow a 50-ml overnight culture of XL1-Blue MRF′ cells in LB broth with supplements at 30°C with shaking.

Day 2

2. Gently spin down the XL1-Blue MRF′ cells (1000 × *g*). Resuspend the cell pellet in 25 ml of 10 mM $MgSO_4$. Measure the OD_{600} of the cell suspension, then dilute the cells to an OD_{600} of 0.5 in 10 mM $MgSO_4$.

3. Combine aliquots of the packaged mixture or library suspension containing ~5 × 10^4 pfu of bacteriophage with 600 µl of XL1-Blue MRF′ cells at an OD_{600} of 0.5 in 14-ml BD Falcon polypropylene round-bottom tubes. To amplify 1 × 10^6 plaques, use a total of 20 aliquots (each aliquot contains 5 × 10^4 plaques/150-mm plate).

   Note *Do not add more than 300 µl of phage/600 µl of cells.*

4. Incubate the tubes containing the phage and host cells for 15 minutes at 37°C to allow the phage to attach to the cells.

5. Mix 6.5 ml of NZY top agar, melted and cooled to ~48°C, with each aliquot of infected bacteria and spread evenly onto a freshly poured 150-mm NZY agar plate. Allow the plates to set for 10 minutes.

6. Invert the plates and incubate at 37°C for 6–8 hours. Do not allow the plaques to get larger than 1–2 mm. On completion, the plaques should be touching.

7. Overlay the plates with ~8–10 ml of SM buffer. Store the plates at 4°C overnight (with *gentle* rocking if possible). This allows the phage to diffuse into the SM buffer.

Day 3

8. Recover the bacteriophage suspension from each plate and pool it into a sterile polypropylene container. Rinse the plates with an additional 2 ml of SM buffer and pool. Add chloroform to a 5% (v/v) final concentration. Mix well and incubate for 15 minutes at room temperature.

9. Remove the cell debris by centrifugation for 10 minutes at 500 × *g*.

10. Recover the supernatant and transfer it to a sterile polypropylene container. If the supernatant appears cloudy or has a high amount of cell debris, repeat steps 8 and 9. If the supernatant is clear, add chloroform to a 0.3% (v/v) final concentration and store at 4°C. Store aliquots of the amplified library in 7% (v/v) DMSO at –80°C.

11. Check the titer of the amplified library using host cells and serial dilutions of the library. (Assume ~10^9–10^{11} pfu/ml.)

Note *Briefly spin the lambda phage stock to ensure that the chloroform is separated completely before removing the aliquot for titering.*

PERFORMING PLAQUE LIFTS

1. Titer the amplified mixture or library suspension to determine the concentration using XL1-Blue MRF´ cells.

2. Combine the equivalent of 5 × 10^4 pfu/plate and 600 µl of freshly prepared XL1-Blue MRF´ cells at an OD_{600} of 0.5.

3. Incubate the bacteria and phage mixture at 37°C for 15 minutes to allow the phage to attach to the cells.

4. Add 6.5 ml of NZY top agar (~48°C) to the bacteria and phage mixture.

5. Quickly pour the plating culture onto a dry, prewarmed 150-mm NZY agar plate, which is at least 2 days old. Carefully swirl the plate to distribute the cells evenly. Allow the plates to set for 10 minutes. (Use 20 plates to screen 1 × 10^6 pfu.)

6. Invert the plates and incubate at 37°C for ~8 hours.

7. Chill the plates for 2 hours at 4°C to prevent the top agar from sticking to the nitrocellulose membrane.

Note *Use forceps and wear gloves for the following steps.*

8. Place a nitrocellulose membrane onto each NZY agar plate for 2 minutes to allow the transfer of the phage particles to the membrane. Use a needle to prick through the membrane and agar for orientation. (If desired, waterproof ink in a syringe needle may be used.)

   Notes *If making duplicate nitrocellulose membranes, allow the second membrane to transfer for ~4 minutes.*

   Pyrex® dishes are convenient for the following steps. All solutions should be at room temperature.

   a. Denature the nitrocellulose-bound DNA after lifting by submerging the membrane in a 1.5 M NaCl and 0.5 M NaOH denaturation solution for 2 minutes.

   Note *If using charged nylon, wash with gloved fingertips to remove the excess top agar.*

   b. Neutralize the nitrocellulose membrane for 5 minutes by submerging the membrane in a 1.5 M NaCl and 0.5 M Tris-HCl (pH 8.0) neutralization solution.

   c. Rinse the nitrocellulose membrane for no more than 30 seconds by submerging the membrane in a 0.2 M Tris-HCl (pH 7.5) and 2× SSC buffer solution (see *Preparation of Media and Reagents*).

9. Blot briefly on a Whatman® 3MM paper.

10. Crosslink the DNA to the membranes using the autocrosslink setting on the Stratalinker UV crosslinker* (120,000 µJ of UV energy) for ~30 seconds. Alternatively, oven-bake at 80°C for ~1.5–2 hours.

11. Store the stock agar plates of the transfers at 4°C to use after screening.

* Available from Stratagene Products Division, Catalog #400071 (1800) and #400075 (2400).

HYBRIDIZING AND SCREENING

Following the preparation of the membranes for hybridization, perform prehybridization, probe preparation, hybridization, and washes for either oligonucleotide probes or double-stranded probes and then expose the membranes to film as outlined in standard methodology texts.[6, 11] Following these procedures, perform secondary and tertiary screens also as outlined in the standard methodology texts.[6, 11] When using the ZAP Express vector, perform in vivo excision on the isolates to obtain the insert-containing pBK-CMV phagemid vector (see *In Vivo Excision of the pBK-CMV from the ZAP Express Vector* and *In Vivo Excision Protocols Using ExAssist Helper Phage with XLOLR Strain*). For clones that are toxic after excision and cannot be propagated as high-copy number plasmids, an alternative is to isolate DNA from the clone as recombinant lambda DNA. In this case, after the phage isolate is obtained, prepare phage DNA using the Stratagene Lambda DNA Purification Kit (Catalog #200391) or using protocols provided in Reference 11.

ANTIBODY SCREENING IN *Escherichia coli*

A complete instruction manual for immunoscreening is supplied with the Stratagene *pico*Blue immunoscreening kit [Catalog #200371 (goat anti-rabbit) and #200372 (goat anti-mouse)]. This kit is available with goat anti-rabbit antibodies or goat anti-mouse antibodies.

EUKARYOTIC SCREENING WITH THE ZAP EXPRESS LIBRARY

Screening libraries in eukaryotic cells has proved to be a very effective way of identifying clones that are not possible to identify using prokaryotic screening systems. The screening technique used will depend on the clone of interest and on the type of assay available. An appropriate cell line for screening must be obtained, and an assay or reagent capable of identifying the cell or cells expressing the desired target protein must be developed. Three different techniques are available: selection, panning, and functional analysis of clone pools.

Selective Assay

Devising a selective assay for eukaryotic library screening requires a cell line that can grow in nonselective media and where expression of a transfected gene permits growth in selective media. An example of this method is screening for a thymidine kinase (TK) gene in L-TK$^-$ cells. If TK$^-$ cells are grown in HAT media, only those cells transfected with a clone coding for a protein capable of replacing TK will grow.

Panning Assay

Clone identification by "panning" requires the transfection of a library into a cell line deficient in the desired surface protein. When the clone of interest is translated and expressed on the surface of eukaryotic cells, the translated protein product is made accessible to an antibody, ligand, or receptor coupled either directly or indirectly to a solid-phase matrix. Eukaryotic transfectant clones expressing the appropriate insert will bind to the affinity matrix, while cells not adhering are washed away. Either transient or stable transfection protocols can be used.

Functional Assay

Functional assay screening can also be performed on either transient or stably transfected cells. Transient expression will likely require subdividing the amplified library into smaller pools of clones to prevent the dilution of a positive cell signal with an excess of negative clones. Each clone pool is amplified separately and transfected into the eukaryotic cells. The transfected cells are then tested for the expression of the desired clone. Once a pool is identified as containing the clone of interest, it is subdivided into smaller pools for a second round of prokaryotic amplification, eukaryotic transfection, and screening. After several rounds of enriching for the desired clone, a single clone can be isolated. The initial pool size is determined according to the sensitivity of the available assay so that a single clone within the pool is still theoretically detectable in the transfected cells. For example, if a positive assay signal is 1000-fold above background, pools containing 500–1000 members should still give a signal above background. The sensitivity of the assay dictates the initial size of the pools, as well as the number of pools required to screen. If stable transformants are created using G418 selection, pools of stable clones can be assayed. This simplifies the identification of isolated positive eukaryotic clones, because the eukaryotic colonies can be picked or diluted in microtiter tissue culture plates.

After a clone has been identified within the eukaryotic cells, the clone can be retrieved by several methods. Plasmid DNA within the tissue culture cells can be collected using the Hirt and Birnboim and Doly procedures,[12, 13] then transferred into *E. coli* cells for amplification and plasmid DNA preparations. Simmons *et al.*[14] were able to screen libraries in COS cells, where the presence of the SV40 T antigen increases the copy number of phagemids containing the SV40 origin of replication. This results in a higher episomal copy number, which may help in the retrieval of the plasmids. Inserts can also be isolated by polymerase chain reaction (PCR) amplification of the tissue culture cells using T3/T7 primer sets. The resulting PCR fragment can be digested using restriction sites flanking the insert, then recloned into pBK-CMV phagemid DNA for further analysis.

Note *Screening libraries in eukaryotic cells can be extremely laborious. Many functional assays are not sensitive enough to detect a clone from pools of nonrelated clones. Therefore, it is worth considering the use of techniques, such as differential PCR,[15] selective hybridization,[16] and degenerate oligonucleotides, to develop DNA probes for initial screening using prokaryotic plaques. Positive clones can then be screened by eukaryotic transfection and expression.*

Protocol

Libraries are constructed in the ZAP Express vector (see *Ligating cDNA into the ZAP Express Vector*; *Packaging*; and *Plating and Titering*), and the libraries are titered to determine size (see *Plating and Titering*). Amplify the library as described in *Amplifying the ZAP Express Library*. Eukaryotic screening can be performed with cesium-banded, double-stranded phagemid DNA prepared from the excised library (see *In Vivo Excision of the pBK-CMV Phagemid Vector from the ZAP Express Vector*). The library can be introduced into the eukaryotic cells as separate pools or as an entire library, depending on the assay system.

EUKARYOTIC EXPRESSION

The CMV promoter is considered to be a strong promoter and to function in many different cell lines.[17] However, expression in eukaryotic cells is sensitive to many factors. If little or no expression is observed in eukaryotic cells, several factors can be considered.

1. For library screening where the insert may not be full length, the *lacZ* ATG allows the expression and detection of fusion protein. However, the 5´-untranslated sequences and amino-terminal fusion can affect expression levels of some inserts. If the clone is identified by prokaryotic screening techniques and is known to be full length (i.e., containing its own ATG and Kozak sequence[18]), the prokaryotic 5´-untranslated sequences may be removed by digesting the excised phagemid with *Nhe* I and *Spe* I, religating the phagemid, then screening for clones which have lost the 200-bp fragment. Other cloning strategies may be used if these sites exist within the insert. Removal of this region may increase expression levels in some inserts. This effect may be due to increased specific activity of the expressed protein by eliminating the expression of fusion proteins, increased RNA stability, or increased translation efficiency by removing the competing upstream *lacZ* ATG.

2. Methylation of some insert DNA can prevent expression in some cell lines.[19]

3. The promoter may not be functional in some cell lines and should be tested before screening a library.

IN VIVO EXCISION OF THE pBK-CMV PHAGEMID VECTOR FROM THE ZAP EXPRESS VECTOR

The ZAP Express vector is designed to allow simple, efficient in vivo excision and recircularization of any cloned insert contained within the lambda vector to form a phagemid containing the cloned insert.[5] This in vivo excision depends on the placement of the DNA sequences within the lambda phage genome and on the presence of a variety of proteins, including filamentous (e.g., M13) bacteriophage-derived proteins. The M13 phage proteins recognize a region of DNA normally serving as the f1 bacteriophage "origin of replication." This origin of replication can be divided into two overlying parts: (1) the site of initiation and (2) the site of termination for DNA synthesis.[20] These two regions are subcloned separately into the ZAP Express vector. The lambda phage (target) is made accessible to the M13-derived proteins by simultaneously infecting a strain of *E. coli* with both the lambda vector and the M13 helper phage.

Inside *E. coli*, the "helper" proteins (i.e., proteins from M13 phage) recognize the initiator DNA that is within the lambda vector. One of these proteins then nicks one of the two DNA strands. At the site of this nick, new DNA synthesis begins and duplicates whatever DNA exists in the lambda vector "downstream" (3´) of the nicking site. DNA synthesis of a new single strand of DNA continues through the cloned insert until a termination signal, positioned 3´ of the initiator signal, is encountered within the constructed lambda vector. The ssDNA molecule is circularized by the gene II product from the M13 phage, forming a circular DNA molecule containing the DNA between the initiator and terminator. In the case of ZAP Express vector, this includes all sequences of the pBK-CMV phagemid vector and the insert, if one is present. This conversion is the "subcloning" step, since all sequences associated with normal lambda vectors are positioned outside of the initiator and terminator signals and are not contained within the circularized DNA. In addition, the circularizing of the DNA automatically recreates a functional f1 origin as found in f1 bacteriophage or phagemids.

Signals for "packaging" the newly created phagemid are linked to the f1 origin sequence. The signals permit the circularized ssDNA to be "packaged" into phagemid particles and secreted from the *E. coli*. Following secretion of the phagemid particle, the *E. coli* cells used for in vivo excision of the cloned DNA are killed, and the lambda phage is lysed by heat treatment at 70°C. The phagemid is not affected by the heat treatment. *Escherichia coli* is infected with the phagemid and can be plated on selective media to form colonies. DNA from excised colonies can be used for analysis of insert DNA, including DNA sequencing, subcloning, and mapping. Colonies from the excised pBK-CMV phagemid vector can also be used for subsequent production of ssDNA suitable for dideoxy-sequencing and site-specific mutagenesis.

IN VIVO EXCISION PROTOCOLS USING EXASSIST HELPER PHAGE WITH XLOLR STRAIN

The ExAssist helper phage with XLOLR strain is designed to efficiently excise the pBK-CMV phagemid vector from the ZAP Express vector, while eliminating problems associated with helper phage co-infection. The ExAssist helper phage contains an amber mutation that prevents replication of the helper phage genome in a nonsuppressing *E. coli* strain such as XLOLR cells. This allows only the excised phagemid to replicate in the host, removing the possibility of co-infection from the ExAssist helper phage. Since the ExAssist helper phage cannot replicate in the XLOLR strain, single-stranded rescue cannot be performed in this strain using this phage.

Mass excision can be used to generate subtraction libraries and subtracted DNA probes. Converting the library to the phagemid form also allows screening of the phagemid library in eukaryotic cells by transformation of eukaryotic cells with supercoiled plasmid DNA.[4, 21]

Single-Clone Excision Protocol

Day 1

1. Core the plaque of interest from the agar plate and transfer the plaque to a sterile microcentrifuge tube containing 500 µl of SM buffer and 20 µl of chloroform. Vortex the microcentrifuge tube to release the phage particles into the SM buffer. Incubate the microcentrifuge tube for 1–2 hours at room temperature or overnight at 4°C. (This phage stock is stable for up to 6 months at 4°C.)

2. Grow separate 50-ml overnight cultures of XL1-Blue MRF′ and XLOLR cells in LB broth with supplements at 30°C.

Day 2

3. Gently spin down the XL1-Blue MRF′ and XLOLR cells (1000 × *g*). Resuspend each of the cell pellets in 25 ml of 10 mM $MgSO_4$. Measure the OD_{600} of the cell suspensions, then adjust the concentration of the cells to an OD_{600} of 1.0 (8×10^8 cells/ml) in 10 mM $MgSO_4$.

4. Combine the following components in a 14-ml BD Falcon round-bottom polypropylene tube:

   200 µl of XL1-Blue MRF′ cells at an OD_{600} of 1.0
   250 µl of phage stock (containing $>1 \times 10^5$ phage particles)
   1 µl of the ExAssist helper phage ($>1 \times 10^6$ pfu/µl)

   Note *Briefly spin the lambda phage stock to ensure that the chloroform is separated completely before removing the aliquot used in the excision reaction.*

5. Incubate the BD Falcon polypropylene tube at 37°C for 15 minutes to allow the phage to attach to the cells.

6. Add 3 ml of LB broth with supplements and incubate the polypropylene tube for 2.5–3 hours at 37°C with shaking. Because clonal representation is not relevant, single-clone excision reactions can be safely performed overnight.

   Note *The turbidity of the media is not indicative of the success of the excision.*

7. Heat the BD Falcon polypropylene tube at 65–70°C for 20 minutes to lyse the lambda phage particles and the cells. Spin the tube at 1000 × *g* for 15 minutes to pellet the cell debris.

8. Decant the supernatant into a sterile 14-ml BD Falcon polypropylene round-bottom tube. This stock contains the excised pBK-CMV phagemid packaged as filamentous phage particles and may be stored at 4°C for 1–2 months.

9. To plate the excised phagemids, add 200 µl of freshly grown XLOLR cells from step 3 (OD_{600} = 1.0) to two 1.5-ml microcentrifuge tubes. Add 100 µl of the phage supernatant (from step 8 above) to one microcentrifuge tube and 10 µl of the phage supernatant to the other microcentrifuge tube.

10. Incubate the microcentrifuge tubes at 37°C for 15 minutes.

11. Add 300 µl of NZY broth and incubate the tubes at 37°C for 45 minutes to allow sufficient expression of the kanamycin-resistance gene product prior to plating on selective medium.

12. Plate 200 µl of the cell mixture from each microcentrifuge tube on LB-kanamycin agar plates (50 µg/ml) and incubate the plates overnight at 37°C.

Due to the high-efficiency of the excision process, it may be necessary to titrate the supernatant to achieve single-colony isolation.

Colonies appearing on the plate contain the pBK-CMV double-stranded phagemid vector with the cloned DNA insert. Helper phage will not grow, since helper phage is unable to replicate in Su^- (nonsuppressing) XLOLR strain and does not contain kanamycin-resistance genes. XLOLR cells are also resistant to lambda phage infection, thus preventing lambda phage contamination after excision.

To maintain the pBK-CMV phagemid vector, streak the colony on a new LB–kanamycin agar plate. For long-term storage, prepare a bacterial glycerol stock and store at –80°C.

R408 helper phage is recommended for the single-stranded rescue procedure. (See *Appendix: Recovery of Single-Stranded DNA from Cells Containing the pBK-CMV Phagemid Vector* for the protocol.)

Mass Excision Protocol

Day 1

1. Grow separate 50-ml overnight cultures of XL1-Blue MRF′ and XLOLR cells in LB broth with supplements at 30°C.

Day 2

2. Gently spin down the XL1-Blue MRF′ and XLOLR cells (1000 × *g*). Resuspend each of the cell pellets in 25 ml of 10 mM $MgSO_4$. Measure the OD_{600} of the cell suspensions, then adjust the concentration of the cells to an OD_{600} of 1.0 (8×10^8 cells/ml) in 10 mM $MgSO_4$.

3. In a 50-ml conical tube, combine a portion of the amplified lambda bacteriophage library with XL1-Blue MRF′ cells at a MOI of 1:10 lambda phage-to-cell ratio. Excise 10- to 100-fold more lambda phage than the size of the primary library to ensure statistical representation of the excised clones. Add ExAssist helper phage at a 10:1 helper phage-to-cells ratio to ensure that every cell is co-infected with lambda phage and helper phage.

   For example, use

   - 10^7 pfu of the lambda phage (i.e., 10- to 100-fold above the primary library size)
   - 10^8 XL1-Blue MRF′ cells (1:10 lambda phage-to-cell ratio, noting that an OD_{600} of 1.0 corresponds to 8×10^8 cells/ml)
   - 10^9 pfu of ExAssist helper phage (10:1 helper phage-to-cells ratio)

   Note *Briefly spin the lambda phage stock to ensure that the chloroform is separated completely before removing the aliquot used in the excision reaction.*

4. Incubate the conical tube at 37°C for 15 minutes to allow the phage to attach to the cells.

5. Add 20 ml of LB broth with supplements and incubate the conical tube for 2.5–3 hours at 37°C with shaking.

   Notes *Incubation times for mass excision in excess of 3 hours may alter the clonal representation.*

   The turbidity of the media is not indicative of the success of the excision.

6. Heat the conical tube at 65–70°C for 20 minutes to lyse the lambda phage particles and the cells.

7. Spin the conical tube at 1000 × *g* for 10 minutes to pellet the cell debris and then decant the supernatant into a sterile conical tube.

8. To titer the excised phagemids, combine 1 µl of this supernatant with 200 µl of XLOLR cells from step 2 in a 1.5-ml microcentrifuge tube.

9. Incubate the microcentrifuge tube at 37°C for 15 minutes.

10. Add 40 µl of 5× NZY broth (for a final concentration of 1×) and incubate the tube at 37°C for 45 minutes to allow sufficient expression of the kanamycin-resistance gene product prior to plating on selective medium.

11. Plate 100 µl of the cell mixture onto LB–kanamycin agar plates (50 µg/ml) and incubate the plates overnight at 37°C.

    Note *It may be necessary to further dilute the cell mixture to achieve single-colony isolation.*

Colonies may now be selected for plasmid preps, or the cell mixture may be plated directly onto filters for colony screening.

APPENDIX I: PURIFYING AND QUANTIFYING RNA

Purifying RNA

We highly recommends using the RNA Isolation Kit or the guanidinium thiocyanate–phenol–chloroform extraction method[10] to isolate total RNA. This method is rapid, yet it produces large amounts of high-quality, undegraded RNA.

Although AccuScript RT is not inhibited by ribosomal RNA (rRNA) and transfer RNA (tRNA) contamination, it is advisable to select the poly(A)$^+$ fraction. The amounts of rRNA and tRNA vastly outnumber the mRNA and will decrease the efficiency of the system. Poly(A)$^+$ RNA is selected on oligo(dT) cellulose columns.[22] Some protocols call for the addition of SDS in the purification steps. Sodium dodecyl sulfate is a powerful enzyme inhibitor and helps prevent degradation of the RNA by RNases, but its presence can also inhibit the enzymes required for cDNA synthesis. If the mRNA intended for use with this kit is suspended in an SDS solution, the RNA must be phenol extracted and ethanol precipitated.

Ribonucleases A and T1 are widely used in almost all molecular biology labs and are nearly indestructible. Ribonucleases are produced by microbes and have also been found in the oils of the skin. Make an effort to use tubes and micropipet tips which have been handled only with gloves. Use freshly autoclaved and baked tips and tubes. Usually these precautions are sufficient, but to be absolutely certain that microcentrifuge tubes and other components intended for use with RNA are not contaminated, the components can be treated with DEPC. Diethylpyrocarbonate is extremely toxic and should be handled with care. Submerge the microcentrifuge tubes in a 0.1% (v/v) DEPC-treated water solution. Leave the beaker of submerged tubes in a fume hood overnight and then dispose of the DEPC-treated water. Autoclave the microcentrifuge tubes for at least 30 minutes. Even though the tubes may still have a sweet DEPC odor, the DEPC is completely inactivated by this procedure. Place the tubes in a drying oven overnight. Equipment which cannot be treated by DEPC can be rinsed in a freshly mixed 3% (v/v) hydrogen peroxide solution, followed by a methanol rinse. Remember, once the RNA is converted to first-strand cDNA, RNases are no longer a concern. Caution should still be exercised in maintaining a sterile, DNase-free environment.

Quantifying RNA

RNA can be quantified by measuring the optical density of a dilute RNA solution. The conversion factor for RNA at the wavelength of 260 nm is 40 µg/ml/OD unit as shown in the example below.

Two microliters of a poly(A)$^+$ RNA sample is added to 498 µl of water (e.g., $OD_{260} = 0.1$). Therefore,

$$0.1\,\text{OD unit} \times \left(\frac{500}{2}\ \text{dilution factor}\right) \times 40\ \mu\text{g of RNA / ml} = 1000\ \mu\text{g of RNA / ml or } 1\ \mu\text{g of RNA / µl}$$

If a sample has significant rRNA contamination, the actual amount of mRNA available for cDNA conversion will be overestimated by this procedure.

If the amount of poly(A)$^+$ RNA is below 1.5 µg/synthesis reaction, the RT may synthesize unclonable hairpin structures. If the amount of poly(A)$^+$ RNA is above 7 µg, the percentage of cDNAs which are full length may decrease. The ZAP Express cDNA synthesis kit is optimized for 5 µg of poly(A)$^+$ RNA, but successful libraries have been generated using the minimums and maximums described here.

Some cDNA procedures recommend heating RNA prior to synthesis to remove any inhibiting secondary structures. Tests conducted with the provided test RNA and several different heterogeneous mRNA samples indicate that a preheating step does not improve cDNA synthesis. If the RNA contains even a minute amount of RNase, its activity will increase by several orders of magnitude with the increased temperature and significantly degrade the RNA. Secondary structure may be a problem with certain RNAs, particularly plant and tumor mRNAs. These samples can be treated with methylmercury hydroxide (see *Appendix II: Treating with Methylmercury Hydroxide*).[23] This chemical is extremely toxic and should be used with caution in a fume hood.

Formaldehyde RNA Gel Protocol

Additional Reagents Required

10× MOPS buffer[§]
37% formaldehyde solution
Agarose
Formaldehyde gel loading buffer[§]
Size standards

Agarose gels [1% (w/v)] usually work well for mixed populations of RNA. If a smaller population of RNA is anticipated, 1.5% (w/v) agarose gels are recommended.

RNA size standards work best. DNA size standards can be used to approximate sizes. If rRNA is present, its intact bands can indicate size and intactness of the sample.

[§] See *Preparation of Media and Reagents*.

The following procedure is for minigels:

1. Melt 1 g of agarose in a solution made with the following:

   10 ml of 10× MOPS buffer
   85 ml of sterile water

2. Allow the melted agarose solution to cool to ~50°C.

3. In a fume hood or in a well-ventilated area, add 5.4 ml of 37% (v/v) formaldehyde.

4. Mix the agarose solution by swirling and pour the solution into the gel mold. While the gel is solidifying, dry the RNA samples >2 µl in volume in a vacuum evaporator. Dilute an appropriate amount of 10× MOPS buffer to 1× to be used as running buffer. When the gel is submerged in 1× MOPS running buffer and everything is completely ready, resuspend the dry samples (or RNA in a volume of <2 µl) in 10 µl of formaldehyde gel loading buffer. Heat the sample for 5–10 minutes at 65°C and then load the gel.

5. Run the gel at 5 V/cm. The ethidium bromide (EtBr) will migrate to the negative electrode and the bromophenol blue (BPB) will travel to the positive electrode.

Usually when BPB has run half the distance of the gel, the RNA has migrated sufficiently to allow the examination of the size distribution relative to the standards. Examine the gel under ultraviolet (UV) illumination. Typical eukaryotic RNA has a majority of its size distribution between 400 and 2000 bases.

Northern blots can easily be produced by soaking the gels in two changes of 10× SSC buffer (for 20 minutes each time) to remove the formaldehyde from the gel and then by blotting the gel either conventionally[11] or by pressure blotting with the PosiBlot 30–30 pressure blotter and Pressure Control Station. The RNA is permanently bound to the nitrocellulose or nylon membrane by conventional baking in an 80°C vacuum oven for 2 hours or by crosslinking for 30 seconds in a Stratalinker UV crosslinker. The blot is probed using conventional methods.[11]

APPENDIX II: TREATING WITH METHYLMERCURY HYDROXIDE

Warning *Methylmercury hydroxide is an extremely toxic chemical. Wear gloves and use with caution in a fume hood.*

1. Resuspend the mRNA in 20 µl of DEPC-treated water.
2. Incubate at 65°C for 5 minutes.
3. Cool to room temperature.
4. Add 2 µl of 100 mM CH_3HgOH.
5. Incubate at room temperature for 1 minute.
6. Add 4 µl of 700 mM β-mercaptoethanol (see *Preparation of Media and Reagents*).
7. Incubate at room temperature for 5 minutes.

APPENDIX III: ALKALINE AGAROSE GELS

Alkaline agarose gels cause DNA to denature and can be used to identify the presence of a secondary structure called hairpinning. Hairpinning can occur in either the first- or second-strand reactions when the newly polymerized strand "snaps back" on itself and forms an antiparallel double helix.

Denaturing gels such as alkaline agarose gels can reveal this secondary structure and can demonstrate the size range of the first- and second-strand cDNA.

Note *The test cDNA sample will run as a tight band at 1.8 kb and will show distinctly different intensity between the first and second strands. This is due to the relative ratio of* α-^{32}P *to the amount of NTP in the first- or second-strand reaction. Normally the second strand will be only 1/10 to 1/20 the intensity of the first-strand band.*

Alkaline agarose gels differ from conventional gels in the following ways:

1. The absence of any buffering capacity in the "buffer" reduces the speed at which the sample can be run.
2. The thickness of the typical undried agarose gel causes the radioactive emissions to be scattered to a degree which makes autoradiographs of the gel difficult to interpret.

The following alternative methods help avoid these complications.

The Slide Technique

The easiest and least expensive method is to use a 5- × 7.5-cm glass slide, position a minigel comb over it with high tension clips, and add 10 ml of molten alkaline agarose near the upper center of the slide. The surface tension of the solution will prevent overflow and produce a small, thin gel which can be exposed to film without further drying. Do not allow the teeth of the comb to overlap the edge of the plate or the surface tension may be broken. To improve the resolution, pat the gel dry with several changes of Whatman 3MM paper after electrophoresis is complete.

To prevent radioactive contamination of film cassettes, seal the wet gels in airtight hybridization bags. Be careful not to trap any air in the hybridization bag which could lift the film away from the gel and cause blurring.

The Vertical Alkaline Agarose Technique

Vertical alkaline agarose gels can be produced using a vertical gel apparatus with 1.5-mm spacers. Since the alkaline agarose gels do not have sufficient friction to remain bound to ordinary glass, a frosted glass plate or gel bond must be used with the vertical apparatus. The combs normally used for acrylamide can be used with this apparatus, if the outside teeth are wrapped in tape to prevent the comb from sinking more than 1.2 cm into the agarose. The 55°C agarose will solidify almost immediately on contact with the cold glass plates, so it is essential to load the mold rapidly with a 60-ml syringe. The comb should already be in the mold, and if it is necessary to reposition the comb, do it immediately after the gel is poured. In order to reduce the possibility of destroying the wells when pulling out the comb, place the solidified gel in a –20°C freezer for 5 minutes immediately prior to removing the comb. When pulling out the comb, it is essential to avoid a vacuum between the teeth and the well. Vacuum can be detected when the well distorts from its normal square shape. When a vacuum occurs, push the comb to separate the glass plates and break the vacuum. After the samples have been run and the glass plates are ready to be opened, slide the unfrosted glass plate off of the alkaline agarose gel instead of prying the plate away from the gel. Pat the gel dry several times using several pieces of Whatman 3MM paper.

Note *To prevent radioactive contamination of film cassettes, seal the wet gels in airtight hybridization bags. Be careful not to trap any air in the hybridization bag which could lift the film away from the gel and cause blurring.*

Conventional Submerged Gels

These gels will require drying either by blotting or through the use of a gel dryer.

Caution ***Even when multiple layers of absorbent paper are placed under the gel, free nucleotides can easily contaminate the drying apparatus. These gels should be poured as thin as possible and should be dried without heat, if time permits, and should never be dried above 40°C.***

Protocol

Additional Reagents Required

Agarose
10× alkaline buffer[§]
Alkaline agarose 2× loading buffer[§]

The following formula makes 80 ml of 1% (w/v) alkaline agarose for cDNAs in the 1- to 3-kb size range.

Melt 0.8 g of agarose in 72 ml of water. Allow the agarose to cool to 55°C. During this time, assemble the gel apparatus. Add 8 ml of 10× alkaline buffer to the cooled agarose, swirl to mix, and pour the agarose immediately. If buffer is added before the correct temperature is reached, the agarose may not solidify.

Load the sample in an equal volume of alkaline agarose 2× loading buffer. Run the gel with 1× alkaline buffer at 100 mA and monitor the system for heat. If the apparatus becomes warmer than 37°C, the amperage should be reduced. The migration of the BPB in alkaline agarose is similar to the migration in regular agarose and should be run to at least one-half or three-quarters distance of the gel.

Note *The alkali conditions causes the blue dye to fade.*

[§] See *Preparation of Media and Reagents*.

APPENDIX IV: ETHIDIUM BROMIDE PLATE ASSAY—QUANTITATING THE cDNA

An accurate quantitation of DNA can be obtained by UV visualization of samples spotted on EtBr agarose plates. DNA samples of known concentration are prepared for use as comparative standards in this assay.

Preparing the Ethidium Bromide Plates

Note *Prepare the EtBr plates under a fume hood.*

Prepare 100 ml of 0.8% (w/v) agarose using Tris-acetate (TAE) buffer (see *Preparation of Media and Reagents*). Cool the molten agarose to 50°C and then add 10 µl of EtBr stock solution (10 mg/ml). The EtBr stock solution is prepared in distilled water (dH_2O) and is stored in the dark at 4°C. Swirl to mix the EtBr stock solution and pour the solution into 100-mm petri dishes using ~10 ml/plate. Allow the plates to harden and incubate the plates at 37°C to dry, if necessary. These plates may be stored in the dark at 4°C for up to 1 month.

Preparing the Standards

Using a DNA sample of known concentration, make seven serial dilutions in 100 mM EDTA to cover the range from 200 to 10 ng/µl. These standards may be stored at –20°C for 3 months.

Plate Assay for Determination of DNA Concentration

Using a marker, label the petri dish to indicate where the sample and the standards (200, 150, 100, 75, 50, 25, and 10 ng/µl) will be spotted.

Thaw the standards and carefully spot 0.5 µl of each standard onto the surface of a prepared EtBr plate. Be careful not to dig into the surface of the plate. Let capillary action pull the small volume from the pipet tip to the plate surface and do not allow a bubble to form. Change pipet tips between each standard.

After spotting all of the standards, immediately spot 0.5 µl of the cDNA sample onto the plate adjacent to the line of standards. Allow all spots to absorb into the plate for 10–15 minutes at room temperature. Remove the lid and photograph the plate using a UV lightbox. Compare the spotted sample of unknown concentration with the standards.

Do not reuse the plates.

Standards and unknowns must be spotted within 10 minutes of each other.

APPENDIX V: RECOVERY OF SINGLE-STRANDED DNA FROM CELLS CONTAINING THE pBK-CMV PHAGEMID VECTOR

The pBK-CMV vector is a phagemid that can be secreted as single-stranded DNA (ssDNA) in the presence of M13 helper phage. These phagemids contain the intergenic (IG) region of a filamentous f1 phage. This region encodes all of the *cis*-acting functions of the phage required for packaging and replication. In *E. coli* with the F^+ phenotype (containing an F´ episome), pBK-CMV phagemid vectors will be secreted as single-stranded f1 "packaged" phage when the bacteria have been infected by a helper phage. Because these filamentous helper phages (M13, f1) will not infect *E. coli* without an F´ episome coding for pili, **it is essential to use the XL1-Blue MRF´ strain or a similar strain containing the F´ episome.**[24, 25]

The Stratagene Products Division offers helper phage that *preferentially* package the pBK-CMV phagemid vector. Typically, 30–50 pBK-CMV molecules are packaged per helper phage DNA molecule. The pBK-CMV phagemid vector is offered with the IG region in the minus orientation.

Yields of ssDNA can depend on the specific insert sequence, but for most inserts >1 µg of ssDNA can be obtained from a 1.5-ml miniprep if grown in XL1-Blue MRF´. A faint single-stranded helper phage band may appear on a gel at ~4 kb for R408 helper phage. This DNA mixture can be sequenced with primers that are specific for the pBK-CMV phagemid vectors and do not hybridize to the helper phage genome.

R408 helper phage can be used to produce a large amount of single-stranded pBK-CMV phagemid vector. We recommend the ExAssist interference-resistant helper phage with XLOLR strain for the excision of the pBK-CMV phagemid vector from the ZAP Express vector and the R408 helper phage for single-stranded rescue.

Single-Stranded Rescue Protocol

1. Inoculate a single colony into 5 ml of 2× YT broth[§] containing 50 µg/ml kanamycin and R408 helper phage at 10^7–10^8 pfu/ml (MOI ~10).

2. Grow the culture at 37°C with vigorous aeration for 16–24 hours, or until growth has reached saturation.

3. Centrifuge 1.5 ml of the cell culture for 5 minutes in a microcentrifuge.

4. Remove 1 ml of the supernatant to a fresh tube, then add 150 µl of a solution containing 20% PEG8000 and 2.5 M NaCl. Allow phage particles to precipitate on ice for 15 minutes.

   Note *For increased yield, perform the PEG precipitation overnight at 4°C.*

5. Centrifuge for 5 minutes in a microcentrifuge. (A pellet should be obvious.)

6. Remove supernatant. Centrifuge the PEG pellets a few seconds more to collect residual liquid, then remove and discard the residual liquid.

7. Resuspend the pellet in 400 µl of 0.3 M sodium acetate (pH 6.0) and 1 mM EDTA by vortexing vigorously.

8. Extract with 1 volume phenol–chloroform and centrifuge for 1–2 minutes to separate phases.

9. Transfer the aqueous phase to a fresh tube and add 1 ml of ethanol. Centrifuge for 5 minutes.

10. Remove ethanol and dry the DNA pellet.

11. Dissolve the pellet in 25 µl of TE buffer.[§]

12. Analyze 1–2 µl on an agarose gel.

[§] See *Preparation of Media and Reagents.*

TROUBLESHOOTING

Observations	Suggestions
Poor first-strand synthesis	Always mix and spin the enzymes in a microcentrifuge immediately before use. Vortex the buffers vigorously until no precipitate is visible.
	Ensure that AccuScript RT activity is not inhibited. Minute amounts of SDS or lithium in the RNA will inhibit the first-strand synthesis reaction. Do not use these in the RNA preparations. Multiple phenol–chloroform extractions will sometimes remove the inhibitors.
	Ensure that quantity of mRNA is sufficient. Optical density readings may be obscured by contaminating rRNA or DNA and may give a false indication of the amount of mRNA used in the synthesis. Repeat the mRNA preparation.
	Ensure that [α-^{32}P]dNTP is not degraded. If [α-^{32}P]dNTP is heated, is left at room temperature too long, or is contaminated, it may not incorporate into cDNA, giving a false indication that synthesis is not occurring.
Poor second-strand synthesis	Confirm the interpretation of the gel results. Control RNA will show distinctly different intensity between the first and second strand. This is due to the relative amounts of α-^{32}P to the amount of NTP in the first- or second-strand reaction. Normally, the second strand will have only 1/10 to 1/20 the intensity of the first-strand band.
	Always mix and spin the enzymes in a microcentrifuge immediately before use. Vortex the buffers vigorously until no precipitate is visible.
	See the previous suggestions for *Poor first-strand synthesis*.
No first-strand synthesis, but good second-strand synthesis	Ensure that there is no DNA contamination within the RNA preparation.
Hairpinning	Ensure that the incubation temperatures are not higher than 16°C. Add second-strand synthesis reaction components to the first-strand reaction mix on ice and then transfer the reaction mixture directly to 16°C for incubation. After incubation, place the samples on ice immediately.
	Ensure that quantity of mRNA is sufficient. Optical density readings may be obscured by contaminating rRNA or DNA and may give a false indication of the amount of mRNA used in the synthesis. Repeat the mRNA preparation.
	Some sources of RNA may have secondary structure (e.g., tumors, some plants, etc.). The RNA may have to be treated with methylmercury hydroxide to relax the secondary structure (see *Appendix II: Treating with Methylmercury Hydroxide*).
	Ensure that the quantity of DNA polymerase is not too high. Use a calibrated pipet to measure the enzyme. Do not submerge the pipet tip completely in the enzyme solution as additional enzyme will adhere to the outside of the pipet tip.
Low counts in the drip column fractions	The number of counts per second per fraction may vary from 0 to 250 cps and yield primary libraries of $>1 \times 10^6$ pfu. Most of the counts remaining in the drip column are from unincorporated [α-^{32}P]dNTP. Verify the quantity of cDNA on the EtBr plate.
Poor ligation	Ensure that the glycerol concentration is not excessive. Do not use excess ligase. Also, do not submerge the pipet tip completely in the enzyme solution as additional enzyme will adhere to the outside of the pipet tip.

(*table continues on the next page*)

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Observations	Suggestions
The number of colonies is too low	The molar ratios of lambda phage to cells to helper phage is critical. Verify that the titer on the tubes is current and correct and use only calibrated pipettors.
	Excision efficiencies are directly related to the ZAP Express phage titer. If an excision is unsuccessful, prepare a high-titer stock of the phage and repeat the excision procedure.
	The pBK-CMV phagemid vector does not contain ampicillin resistance. Ensure that the platings are performed using kanamycin or neomycin agar plates.
	Chloroform, which is added after packaging to prevent bacterial contamination, lyses the *E. coli* before the helper phage can infect and excise. Be sure to spin down the chloroform completely prior to removing an aliquot of the lambda phage for in vivo excision.

PREPARATION OF MEDIA AND REAGENTS

10× Ligase Buffer

500 mM Tris-HCl (pH 7.5)
70 mM $MgCl_2$
10 mM dithiothreitol (DTT)

Column-Loading Dye

50% (v/v) glycerol
10% (v/v) 10× STE buffer
40% (w/v) saturated BPB[‖]

SM Buffer (per Liter)

5.8 g of NaCl
2.0 g of $MgSO_4 \cdot 7H_2O$
50.0 ml of 1 M Tris-HCl (pH 7.5)
5.0 ml of 2% (w/v) gelatin
Add deionized H_2O to a final volume of 1 liter

20× SSC Buffer (per Liter)

175.3 g of NaCl
88.2 g of sodium citrate
800.0 ml of deionized H_2O
Adjust to pH 7.0 with a few drops of 10 N NaOH
Add deionized H_2O to a final volume of 1 liter

10× STE Buffer

1 M NaCl
200 mM Tris-HCl (pH 7.5)
100 mM EDTA

10× Alkaline Buffer (per 50 ml)

3 ml of 5.0 M NaOH
2 ml of 0.5 M EDTA
45 ml of deionized H_2O

Formaldehyde Gel Loading Buffer

720 µl of formamide
160 µl of 10× MOPS buffer
260 µl of 37% formaldehyde
100 µl of sterile water
100 µl of EtBr (10 mg/ml)
80 µl of sterile glycerol
80 µl of saturated BPB[‖] in sterile water

Note *The formaldehyde gel loading buffer is not stable and should be made fresh on the day of use*

5% Nondenaturing Acrylamide Gel

Mix the following in a vacuum flask

5 ml of 10× TBE buffer
8.33 ml of a 29:1 acrylamide–bis-acrylamide solution
36.67 ml of sterile deionized H_2O

De-gas this mixture under vacuum for several minutes
Add the following reagents
25 µl of TEMED
250 µl of 10% ammonium persulfate

Alkaline Agarose 2× Loading Buffer

200 µl of glycerol
750 µl of water
46 µl of saturated BPB[‖]
5 µl of 5 M NaOH

10× MOPS Buffer

200 mM 3-[*N*-morpholino]propane-sulfonic acid (MOPS)
50 mM sodium acetate
10 mM EDTA
Adjust to a final pH of 6.5–7.0 with NaOH
Do not autoclave

700 mM β-Mercaptoethanol

5 µl of 14 M β-mercaptoethanol
95 µl of DEPC-treated water

1× TAE Buffer

40 mM Tris-acetate
1 mM EDTA

[‖] To make saturated bromophenol blue (BPB), add a small amount of BPB crystals to water and vortex. Centrifuge the sample briefly and look for the presence of an orange pellet. If a pellet is seen, the solution is saturated. If not, add more crystals and repeat the procedure.

LB Broth (per Liter)

10 g of NaCl
10 g of tryptone
5 g of yeast extract
Add deionized H_2O to a final volume of 1 liter
Adjust to pH 7.0 with 5 N NaOH
Autoclave

LB Agar (per Liter)

10 g of NaCl
10 g of tryptone
5 g of yeast extract
20 g of agar
Add deionized H_2O to a final volume of 1 liter
Adjust pH to 7.0 with 5 N NaOH
Autoclave
Pour into petri dishes (~25 ml/100-mm plate)

LB–Tetracycline Agar (per Liter)

Prepare 1 liter of LB agar
Autoclave
Cool to 55°C
Add 1.5 ml of 10 mg/ml filter-sterilized tetracycline
Pour into petri dishes (~25 ml/100-mm plate)
Store plates in a dark, cool place or cover plates with foil if left out at room temperature for extended time periods as tetracycline is light-sensitive

LB–Kanamycin Agar (per Liter)

Prepare 1 liter of LB agar
Autoclave
Cool to 55°C
Add 6.6 ml of 7.5 mg/ml filter-sterilized kanamycin
Pour into petri dishes (~25 ml/100-mm plate)

LB Broth with Supplements

Prepare 1 liter of LB broth
Autoclave
Add the following filter-sterilized supplements prior to use
10 ml of 1 M $MgSO_4$
3 ml of a 2 M maltose solution or
10 ml of 20% (w/v) maltose

NZY Agar (per Liter)

5 g of NaCl
2 g of $MgSO_4 \cdot 7H_2O$
5 g of yeast extract
10 g of NZ amine (casein hydrolysate)
15 g of agar
Add deionized H_2O to a final volume of 1 liter
Adjust the pH to 7.5 with NaOH
Autoclave
Pour into petri dishes (~80 ml/150-mm plate)

NZY Broth (per Liter)

5 g of NaCl
2 g of $MgSO_4 \cdot 7H_2O$
5 g of yeast extract
10 g of NZ amine (casein hydrolysate)
Add deionized H_2O to a final volume of 1 liter
Adjust the pH to 7.5 with NaOH
Autoclave

NZY Top Agar (per Liter)

Prepare 1 liter of NZY broth
Add 0.7% (w/v) agarose
Autoclave

Super Broth (per Liter)[||]	2× YT Broth (per Liter)
35 g of tryptone	10 g of NaCl
20 g of yeast extract	10 g of yeast extract
5 g of NaCl	16 g of tryptone
Add deionized H_2O to a final volume of 1 liter	Add deionized H_2O to a final volume of 1 liter
Adjust to pH 7.5 with 5 M NaOH	Adjust to pH 7.5 with NaOH
Autoclave	Autoclave

[||] LB broth is the medium of choice for overnight growth. However, when growing XL1-Blue MRF´ for in vivo excision, rescue, or minipreps, super broth may be used. Growing host cells overnight plating cultures at 30°C also increases plating efficiency.

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